DEVELOPMENT OF IMPROVED EXPRESSION VECTORS AND THEIR APPLICATIONS IN CANCER GENE THERAPY

by

Phoebe Lihong Luo

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Phoebe Lihong Luo

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David T. Harris, Ph.D.	Date 10/3/03
John J. Marchalonis, Ph.D.	Date 10/03/03
Emmanuel Akporiaye, Ph.D.	Date 10/03/03
Harris Bernstein, Ph.D.	Date 10/03/03
Jesse D. Martinez, Ph.D.	Date 10/05/03

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director David T. Harris, Ph.D. Date 10/3/03

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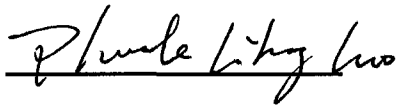
SIGNED: 

Phoebe Lihong Luo

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DEDICATION

This work is dedicated to my parents

Shou-Ren Luo
Yu-Feng Wang

and

All those devoted to find a true cure for diseases

through gene therapy

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ABSTRACT

Recombinant DNA vectors are fundamental tools in gene therapy research. A novel cloning system, pLinus, was made to facilitate vector construction by providing 32 unique restriction sites to adapt DNA fragments in a single step.

To compensate the low delivery efficiency of the non-viral vector systems, we have constructed two high expression plasmid vectors, pHi1/2, by incorporating a transcriptional amplifier strategy into a single construct. In both pHi1/2 vectors, the amplifier expression cassettes contained two independent transcriptional units. One transcriptional unit contained a transcriptional factor, the tat gene, driven by a strong constitutive CMV promoter. The second transcriptional unit contained either an HIV1 LTR or HIV2 LTR driving the gene of interest. Using the human IL-2 cytokine as a reporter and therapeutic gene, the pHi1/2 amplifier vectors could achieve significantly higher IL-2 expression levels than that observed when using the CMV promoter alone. In vivo injection of the stable pHi-2-IL-2 gene modified Lewis Lung (LL/2) tumor clones resulted in slower tumor growth and longer survival as compared to those mice injected with either CMV-driven IL-2 transfected clones or the parental tumor cells.

To solve the safety concern, we constructed a novel plasmid vector, pHi-Hot, by combining inducible and amplifier strategies in a single vector. In pHi-Hot, the first transcriptional unit contained an inducible heat shock protein (hsp70B) promoter controlling the expression of a transcriptional factor, Tat, which trans-

activates a second promoter, the HIV2 LTR, located downstream on the same construct. The second promoter drives the gene of interest. Using the human IL-2 cytokine gene as a reporter gene, we demonstrated that, heat shock at 42°C for 30 min, the pHi-Hot vector could achieve high gene expression levels while maintaining its inducibility. The induced IL-2 levels were significantly higher than achieved by using the hsp promoter or CMV promoter directly. And repeated heat shock at 42°C for 30 min of mice injected with a pHi-Hot-IL-2 gene modified LL/2 clone led to tumor regression.

In this study, three major approaches towards facilitating vector construction and improving vector expression cassette design are described.

CHAPTER 1

INTRODUCTION

1.1 Literature Review

Preamble

In the past few decades, gene transfer has become a promising tool for the treatment of many human diseases (1-5). Gene transfer was initially thought of as a means to correct single gene defects in hereditary diseases. In the meantime, cancer has become by far the most important indication for gene therapy, as shown by many clinical trials (3,5,6). The first clinical trial of gene therapy was performed in 1990 in children with adenosine deaminase deficiency (1,3). Since then more than 5000 patients have been treated worldwide in more than 400 clinical protocols (3,5,6). However, clinical efficacy when using a variety of gene therapy approaches has not been convincingly demonstrated in most of the trials conducted so far, while safety concerns have been raised as the consequence of the "Gelsinger Case" in Philadelphia: fatal toxicity in a 18-year-old young man with hereditary liver disease after treatment with a high dose of adenoviral vectors (2,7). The main reasons for the present lack of clinical success in gene therapy are the low efficiency of gene delivery, low levels of transgene expression and the progressive loss of transgene expression in vivo from the currently available vectors (2,5,8,9). It has become obvious that many

technical difficulties need to be overcome before gene therapy can fulfill its therapeutic promises.

Gene Delivery Systems

The currently employed gene delivery systems can be divided into two major groups: viral and nonviral (3,9-12). Viral systems are by far the most effective means of gene delivery because of their highly evolved and specialized components. Over 500 gene therapy trials have been completed to date, the majority used viral vectors such as retroviruses and adenoviruses. Only about 20% of these trials have used non-viral vectors (3,8,10). None of the currently available vectors so far satisfies all the criteria of an ideal gene therapeutic system. Although viral vectors are more efficient, safety concerns from the use of viruses have been brought sharply into focus within the last several years with the death in the "Gelsinger Case" in a gene therapy trial (2,7). In addition, the limitations of viral-mediated delivery include toxicity, restricted targeting of specific cell types, limited DNA carrying capacity, production and packaging problems, potential recombination, and high costs (2,13-15). Furthermore, the toxicity and immunogenicity of viral systems also hamper their routine use in basic research laboratories and in practical applications (8,15-18).

Nonviral plasmid-based gene expression systems represent an attractive in vivo gene transfer strategy because they are simple, lack many of the risks that are

inherent to viral systems, and most notably have the potential to be administered repeatedly (12,19-22). Therefore, nonviral systems have been increasingly used for developing new gene therapy vectors. However, the relatively low efficiency of gene transfer has limited their use (8,11,23-25). The development of plasmid vectors that exhibit high gene expression levels may partially compensate for the inefficiency in gene delivery, which may be sufficient to correct or at least ameliorate certain disease indications. In addition to their advantageous safety profile, a recent report on the comparative activity of retroviral and non-viral (liposomal) gene transfer in a mouse model observed no survival advantage with the use of retroviruses, which has intensified research into non-viral gene delivery systems (26).

Problems in Gene Therapy Vector Construction

Recombinant vectors are fundamental tools in gene therapy and molecular biology research. One of the problems frequently encountered in vector construction is a lack of compatible restriction sites between the vectors and the DNA inserts. The conventional approach to solve this problem is to make the DNA fragments blunt-ended (27-29) or to add linkers or adaptors (30-32) before ligation. However, the efficiency of using blunt-end ligation and linkers/adaptors is low. The addition of linkers or adaptors can be expensive because a new linker/adaptor has to be made for insertion at each new restriction site. Moreover, linkers/adaptors are not commercially available for all restriction sites. As the

polymerase chain reaction (PCR) has become one of the most important techniques in the field of recombinant DNA, introduction of new restriction sites into DNA fragments by PCR has become an alternative method (33-35). However, PCR-directed mutagenesis requires prior knowledge of the flanking sequences of the targeted fragments in order to design the primers and to synthesize the oligonucleotides. It is also time-consuming to characterize the optimal conditions needed for large-scale PCR amplification. The main disadvantages of PCR-generated mutagenesis are: 1) There exists a size limitation on the fragments that can be amplified by PCR. Introduction of new restriction sites by PCR may not be applicable to large DNA fragments. Using standard Taq DNA polymerase, the size limitation for obtaining high fidelity PCR fragments is less than 5 kilobase (kb) (2). Although currently longer and more accurate DNA amplification can be achieved using high-fidelity polymerases with 3'-->5' proofreading exonuclease activity (36,37), amplifying fragments in excess of 20kb in length is still problematic, especially from complex templates such as genomic DNA (33,36). 2) Sequence analysis is required for each PCR amplified fragment (33). Even with high fidelity polymerases, the entire amplified fragment has to be sequenced to insure there are no polymerase-derived mutations. Furthermore, several rounds of sequence analysis are required for any fragments larger than 800bp because the reliable sequence obtained from each sequencing reaction is only about 500-800bp (33). New primers have to be synthesized for each round of sequencing analysis. 3) Primer design and synthesis, as well as

the entire PCR process, has to be repeated if the same DNA fragment needs to be inserted into different restriction sites. Thus, development of new cloning tools capable of providing user-friendly polylinkers is critical.

Gene Expression Levels and Therapeutic Efficacy

A sufficient level of gene expression is often required for therapeutic efficacy. It has been shown that the efficacy of many therapeutic genes is also dose dependent (17,38). Transgene expression can be enhanced considerably through the use of optimized transcriptional regulatory elements, such as strong promoters (e.g. RSV, retroviral long terminal repeats or the immediate/early promoter of CMV) (20,39,40). Thus, many gene therapy vectors use these strong constitutive promoters to achieve high level gene expression (8,18,39). Among these, the CMV promoter has been reported to produce the highest levels of gene expression and therefore has already been used in clinical trials (39,41,42). It also has shown that using introns and strong polyadenylation (PA) signal sequences may further increase transgene expression. In addition to strong promoter/enhancers, these elements have also been frequently incorporated into expression vector design to further enhance transgene expression levels (20,21). Unfortunately, few patients have shown significant clinical responses from the use of any of the currently used gene therapy vectors (3,8,13,18). These data suggest that further increases in gene expression may be needed in order to reach the therapeutic thresholds required for clinical efficacy.

Gene Expression Regulatory Systems and Safety Issues

Safety is an important consideration in the development of gene therapy protocols. Lack of specificity of vectors for tumor targeting is another major problem facing current cancer gene therapy (2,9,13). For safe and effective gene therapy, high enough transgene expression levels must be reached in target tissues whereas such expression in surrounding nontarget cells should be minimized or eliminated. This aspect is particularly important when the delivered gene products are cytotoxic, such as potent therapeutic cytokines like tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12) (43). This need has led to a requirement for developing gene therapy vectors that can restrict the expression of the therapeutic genes to target cells. Furthermore, there is also a requirement that the level and timing of gene expression can be regulated according to therapeutic needs. Tightly controlled mechanisms for targeting and regulating transgene expression would be very useful in expanding the current applications of potent but cytotoxic therapeutic genes. Controlled and restricted genes expression could be achieved by using gene regulatory elements in vector design (44). Therefore, numerous approaches in gene therapy of human cancers are focused on the establishment of tumor-specific or inducible expression vectors allowing the targeted and/or regulated expression of therapeutic genes (45-50).

Tumor Specific Promoters

Tumor specific promoters can potentially solve the safety concerns in gene therapy because they allow spatial control of gene expression within the tumor tissue (47,48). A number of tumor-specific promoters have already been tested in a variety of models. These promoters include the α-fetoprotein (AFP) promoter in hepatocellular carcinoma, the tyrosine promoter in melanoma and the prostate-specific antigen (PSA) promoter in prostate cancer (51-53). However, the main obstacles in using tumor-specific promoters are the low efficiency of transcriptional activity and the requirement to identify tumor-specific promoters for each type of tumor. In addition, there is often a lack of a method to regulate gene expression driven by these promoters (44,46,48).

Inducible Promoters

Inducible promoters have become alternatives for safe gene therapy by providing temporal control of gene expression. Various inducible systems have been developed in the past few decades. Among these, the tetracycline expression and the radiation inducible systems have been widely used (45,48,50). The advantage of using the tetracycline expression system is that the induction of gene expression can be conveniently controlled by the exogenous antibiotic, tetracycline. However, the main problems when using this system are the low gene expression levels and the toxic levels of transactivator needed to induce high gene expression. Side effects such as anorexia, nausea and brownish

discoloration of teeth due to the slow clearance of tetracyclin in the bones in animals caused by high doses of antibiotics over a long time period have been reported (45,54). Radiation-inducible promoters could provide tight spatial and temporal control of gene expression because the ionizing radiation doses can be controlled with great precision in defined tissue volumes. However, these promoters usually exhibit high baseline expression and low inducibility. Further, within a safe and practical radiation dosage, the inducibility of these promoters is relatively low (54,55).

For safe and effective gene therapy, control over the level and duration of gene expression in the targeted tissues will be essential. The development of vectors that can achieve tumor targeting after systemic administration represents a significant challenge that must be met if gene therapy is to have a major impact against common tumor types.

Due to its unique features and properties, the heat shock promoter (hsp) is one of several inducible promoters offering great potential for spatial and temporal control of gene expression (56-60). Many studies have shown that the basal activity of the hsp promoter is low and the inducibility of this promoter is high (56-58). Promoter activity correlates with elevated temperatures (57,58). As levels of transgene expression are critical in determining efficacy, transgene expression under direct control of the hsp promoter may not be sufficient for optimal therapy,

especially at clinically acceptable heating temperatures. In the clinical setting, the temperatures used to induce gene expression should be in a range that will not impair cell viability. Meanwhile, induced gene expression levels at these temperatures should be high enough for effective therapy. Thus, construction of gene delivery vectors capable of producing high levels of gene expression after induction is required for effective therapy.

Cancer Gene Therapy

The application of gene therapy to cancer has proceeded from the same rational basis as was originally conceptualized for inherited genetic disorders. More than 70% of patients treated to date in human clinical gene therapy protocols have been in the context of anticancer regimens (3,5,6). Strategies for cancer gene therapy include 'suicide' gene therapy using enzyme/prodrug systems, transfer of tumor suppressor genes, inhibition of activated oncogenes by antisense mechanisms, and immuno-gene therapy by cytokine gene transfer and tumor cell vaccination (61-68). Despite anecdotal reports of therapeutic responses in many patients, unequivocal proof of the clinical efficacy of any of the varied approaches to gene therapy of human cancer is still lacking (2,3,8). One of the main impediments to the potential success of gene therapy is the fact that cancer is a disease of many sequential acquired mutations, which may or may not show a particular hierarchy in causing and maintaining malignant transformation (3,15). It is therefore unlikely that the correction of a single gene defect will be sufficient to

reverse this process in the majority of cancer cells within a given tumor. Furthermore, the technical hurdles in gene delivery and expressions in vivo with the currently available vectors may explain the present lack of clinical success with cancer gene therapy (3,69).

Immunopotentiation by Cytokines for Cancer Therapy

The concept of immunological tumor therapy has been studied for more than a hundred years (3,70,71). Recently, gene therapy approaches have brought new optimism to the field of tumor immunology, and both immune effector cells and tumor cells have been identified as possible targets for gene transfer. Strategies such as using cytokines to improve the efficacy of immune effector cells and increasing immunogenicity of tumor cells have been widely used (69-72).

Cytokines are a heterogenous group of cell regulators which are produced by a wide variety of cells in the body (70-73). They are involved in immunity and inflammation where they regulate the amplitude and duration of the response, and are usually produced transiently and locally, acting in a paracrine or autocrine manner (70-72). Cytokines can be useful in cancer treatment by (a) exerting direct effects on a tumor (cytolysis, cytostasis, vascular damage, terminal differentiation); (b) enhancing the expression of major histocompatibility complex (MHC) class I/II antigens, cell adhesion molecules, and other surface moieties on tumor cells including tumor-associated antigens; (c) recruiting,

expanding, and stimulating endogenous effector cells; and (d) maintaining adoptively transferred lymphocyte populations (70-75).

Cytokine therapy for cancer treatment was initially tested by the use of recombinant proteins (73-76). Systemic administration of cytokines like interleukin-2 (IL-2), interferons (IFNs) and TNF-α have been found to be at least partially effective against a wide spectrum of experimental tumors; whereas in patients, primarily IL-2 and IFN-α were beneficial and only in a limited number of malignanices (73-77). However, problems such as insufficient levels of cytokines local to the tumor, the short half-life of cytokines and severe side-effects were found when administered systemically (16,38,75-77).

Gene-Modified Tumor Cells

The toxicity accompanied by in vivo systemic administration of the recombinant cytokine proteins has hampered their extensive clinical application. Therefore, cytokine gene-modified tumor cells, in which the immunizing tumor cells, after cytokine gene transfer, serve as the in vivo cytokine producers, appears to be a promising approach to overcome the problems caused by systemic administration. Many cytokine genes such as IL-2, IL-12, IFN-γ, TNF-α, and granulocyte macrophage-colony stimulating factor (GM-CSF) have been introduced into tumor cells with varying effects on both tumorigenicity and immunogenicity (3,71,77-80). When injected intratumorally, subcutanenously or

by other routes, these manipulated tumor cells are capable of producing high local amounts of the respective protein without causing the systemic side-effects (77-80). This paracrine physiology much more closely mimics the natural biology of cytokine action than does the systemic administration of recombinant cytokines. Animal studies have shown that immunization of immunocompetent mice with transduced tumor cells that secrete IL-2 or IFN-γ could elicit a strong, tumor-specific cellular response. This immune response was more potent and longer lasting than that induced by the unmodified tumor cell vaccine (37,71,78). Gene modified tumor cells are therefore considered less tumorigenic than untransduced parental cells, and are frequently used as live, unirradiated cells for animal vaccination (71,77-80).

In addition to cytokine genes, MHC molecules, viral antigens and tumor antigens such as MAGE genes in melanoma or co-stimulatory molecules such as B7.1 or B7.2, have also been used to increase the immunogenicity of tumor cells in animal models and clinical studies (81-85).

1.2. Statement of purpose of studies:

Gene therapy represents one of the most potentially important developments in oncology. However, before this potential can be realized as a standard treatment, technical problems in gene delivery, efficiency and safety must be overcome. Since the success of gene therapy is largely dependent on the development of

the gene delivery vector, in the following chapters, we have focused on describing the development of new methods and strategies to solve some of these problems in order to improve the safety and efficacy of cancer gene therapy.

Specific Aims:

The specific aims of the studies are to:

1) Develop high efficiency cloning systems to facilitate gene therapy vector construction.
2) Develop high gene expression vectors by incorporating a transcriptional amplifier strategy.
3) Determine the in vitro transgene expression levels of the amplifier vectors.
4) Determine the in vivo tumorigenicity of amplifier vector-modified tumor cell clones.
5) Develop high and controlled transgene expression vectors by incorporating inducible and amplifier strategies.
6) Determine the basal and inducible transgene expression levels of the inducible amplifier vector in vitro.
7) Determine the in vivo tumorigenicity of the inducible amplifier expression vector-modified tumor cell clones.

The ultimate goal of this project is to improve both the efficacy and safety of gene therapy. The strategies discussed here and the results from this study should have significant implications for designing gene expression vectors for human gene therapy. Also, the results should provide insight into possible approaches to overcome current limitations in gene therapy technology.

CHAPTER 2

DEVELOPMENT OF HIGH EFFICIENCY CLONING SYSTEM TO FACILITATE GENE THERAPY VECTOR CONSTRUCTION

2.1 MATERIALS AND METHODS

Restriction endonucleases

Restriction endonucleases were obtained from either New England BioLab (New England BioLab, Beverly, MA, USA) or Roche (Roche Molecular Biochemicals, Indianapolis, Indiana).

DNA ligase

T4 DNA ligase was used from the Rapid DNA Ligation Kit (Roche Molecular Biochemicals, Indianapolis, Indiana).

Construction of the pLinus16-Sal plasmid

The pLitmus 38 and 39 plasmids (New England BioLab, Beverly, MA, USA, see Appendix A) were double-digested with AlwN I and Sal I restriction endonucleases. Lambda DNA was digested with Sal I. Each digested DNA was extracted with an equal volume of phenol and chloroform, precipitated by adding sodium acetate (NaOAc) and ethanol, washed in ethanol, dissolved in 20μl Tris-EDTA (TE) and electrophoresed in 1% low-melting agarose gel containing TAE buffer (See Appendix C). The resulting 2250-bp AlwN I-Sal I fragment of Litmus

39, the 685-bp AlwN I-Sal I fragment of Litmus 38 and the 499-bp Sal I fragment from Lambda DNA were excised from the gel, extracted with the Agarose Gel DNA Extraction Kit (Roche Molecular Biochemicals, Indianapolis, Indiana) and eluted in TE. The 2250-bp AlwN I-Sal fragment, the 685-bp AlwN I-Sal I fragment and the 499-bp Sal I Lambda fragment were added at a 1:1:5 ratio in a final volume of 20μl ligation mixture with 1ul T4 ligase to construct the pLinus16-Sal plasmid (Figure 1A)

Construction of the pLinus16-Apa plasmid

Similarily, pLitmus 38 and 39 plasmids were double-digested with AlwN I and Apa I restriction enzymes. pCMV-EGFP derived from pcDNA3.1 (Invitrogene, Carlsbad, CA, See Appendix B) was digested with the Apa I enzyme. Each digested DNA was extracted, precipitated, washed, dissolved in 20μl TE and then electrophoresed in 1% low-melting agarose gel. The resulting 2250-bp AlwN I-Apa I fragment of pLitmus 38, the 685-bp AlwN I-Apa I fragment of pLitmus 39 and the 780-bp Apa I fragment (EGFP sequence) from pCMV-EGFP were extracted and eluted in TE. The DNA pieces were added at a 1:1:5 ratio respectively, in 20μl ligation mixture with 1μl T4 ligase to construct the pLinus16-Apa plasmid (Figure 1B).

Construction of the pLinus17-Kpn plasmid

The pLitmus 28 and 29 plasmids (New England BioLab, Beverly, MA, USA, see

Appendix A) were double-digested with AlwN I and Kpn I restriction enzymes. Lambda DNA was digested with Kpn I enzyme. Using the same method described above, each digested DNA was extracted, precipitated, washed, dissolved in 20μl TE and electrophoresed in 1% low-melting agarose gel. The resulting 2250-bp AlwN I-Kpn I fragment of pLitmus 29, the 685-bp AlwN I-Kpn I fragment of pLitmus 28 and the 1503-bp Kpn I fragment from Lambda DNA were extracted and eluted in TE and then added at a 1:1:5 ratio in 20μl ligation mixture with 1μl T4 ligase to construct the pLinus17-Kpn plasmid (Figure 2A).

Construction of the pLinus17-Bgl plasmid

The pLitmus 28 and 29 plasmids were double-digested with AlwN I and Bgl II restriction enzymes. Lambda DNA was digested with the Bgl II enzyme. Using the method described above, the digested DNA were extracted, precipitated, washed, dissolved in 20μl TE and electrophoresed in 1% low-melting agarose gel. The resulting 2250-bp AlwN I-Bgl II fragment of pLitmus 28, the 685-bp AlwN I-Kpn I fragment of pLitmus 29 and the 651-bp Bgl II fragment from Lambda DNA were extracted and eluted in TE and then added at 1:1:5 ratio in 20μl ligation mixture with 1μl T4 ligase to construct the pLinus17-Bgl plasmid (Figure 2B)

Transformation of competent cells

E. coli cells (DH5α) were made competent with the Z-Competent E. coli Transform Kit (Geno Technology, St. Louis, MO, USA) and were used to

transform the recombinant DNA. 5μl of each ligation mixture was gently mixed with 200μl cells and incubated on ice for 20 min. 100μl from each competent cells mixture was spread on an Ampicillin-agar plate prewarmed at 37°C. Colonies were selected and grown in LB media with Ampicillin. DNA was isolated by the alkaline lysis method (8).

Analysis of the end products

To analyze the resultant DNA, each pLinus plasmid was digested with the restriction enzymes contained in its polylinker and then electrophoresed in 1% agarose gel containing ethidium bromide (Figure 3A and 3B).

2.2 RESULTS

Design and construction of the pLinus16/17 cloning system

One of the problems frequently encountered in vector construction is a lack of compatible restriction sites between the vectors and the DNA inserts. To address this problem, using the above-described protocols, two sets of plasmids, pLinus16 (Figure 1A and 1B) and pLinus17 (Figure 2A and 2B) were constructed. Both pLinus16 and pLinus17 consist of a pair of plasmids derived from pLitmus 28/29 and 38/39 (New England BioLab, Beverly, MA, USA, see Appendix A), respectively. The plasmids provide a total of 29 unique restriction sites with cohesive ends and three restriction sites (SnaB I, EcoR V and Stu I) for blunt-end ligations. Each pLinus plasmid contains two inverted, repeated copies of the multiple cloning sites (MCS) flanking a central stuffer fragment derived from either Lambda DNA or the EGFP sequence. The 780-bp stuffer piece in pLinus 16-Apa was an EGFP gene fragment, cut with restriction endonuclease Apa I from the pcDNA3.1-EGFP plasmid (See Appendix B). The pcDNA3.1-EGFP was constructed by insertion of a 758-bp Kpn I/Not I EGFP fragment from pEGFP-1 (Clontech, Palo Alto, CA) into the Kpn I/Not I sites of pcDNA3.1 (Invitrogen, Carlsbad, CA). The two inverted repeated copies of the MCS in each pLinus vector allow versatile insertion of DNA fragments and provide a cassette such that DNA pieces can be cut out and cloned into other vectors, thus making the

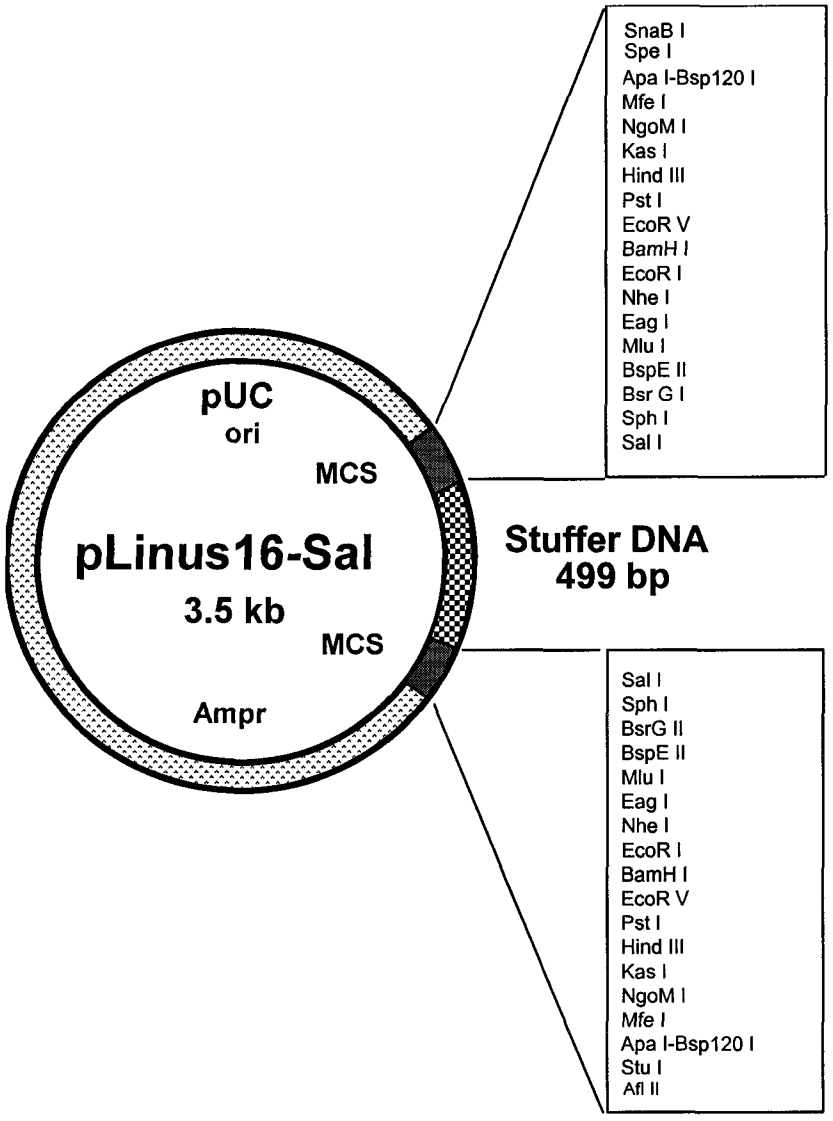


Figure 1A. Diagrammatic representation of the pLinus16-Sal cloning vector. *pLinus16-Sal* was derived by ligating the 2250-bp AlwN I-Sal I fragment of pLitmus 39 with the 685-bp AlwN I-Sal I fragment of pLitmus 38. The stuffer DNA is a 499-bp Sal I fragment derived from Lambda DNA. MCS: multiple cloning sites.

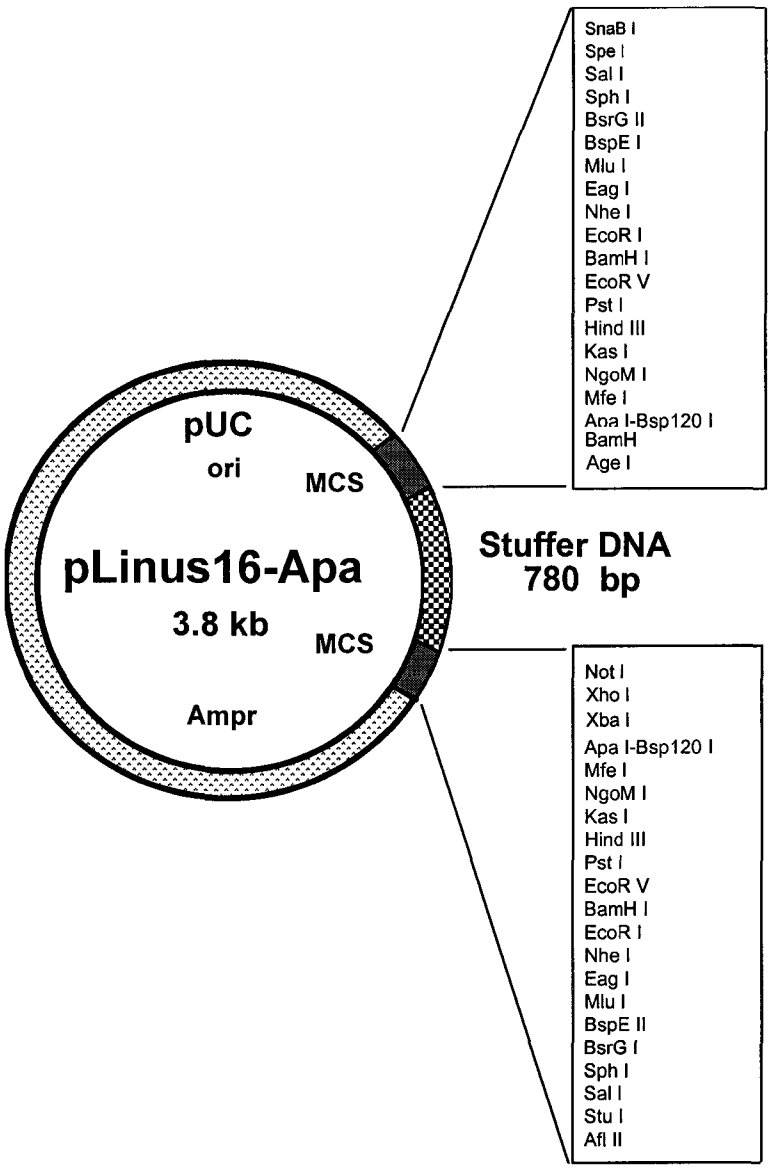


Figure 1B. Diagrammatic representation of the pLinus16-Apa cloning vector. *pLinus16-Apa* was derived by ligating the 2250-bp AlwN I-Apa I fragment of pLitmus 38 with the 685-bp AlwN I-Apa I fragment of pLitmus 39. The stuffer DNA is a 780-bp Apa I fragment containing the EGFP sequence. MCS: multiple cloning sites.

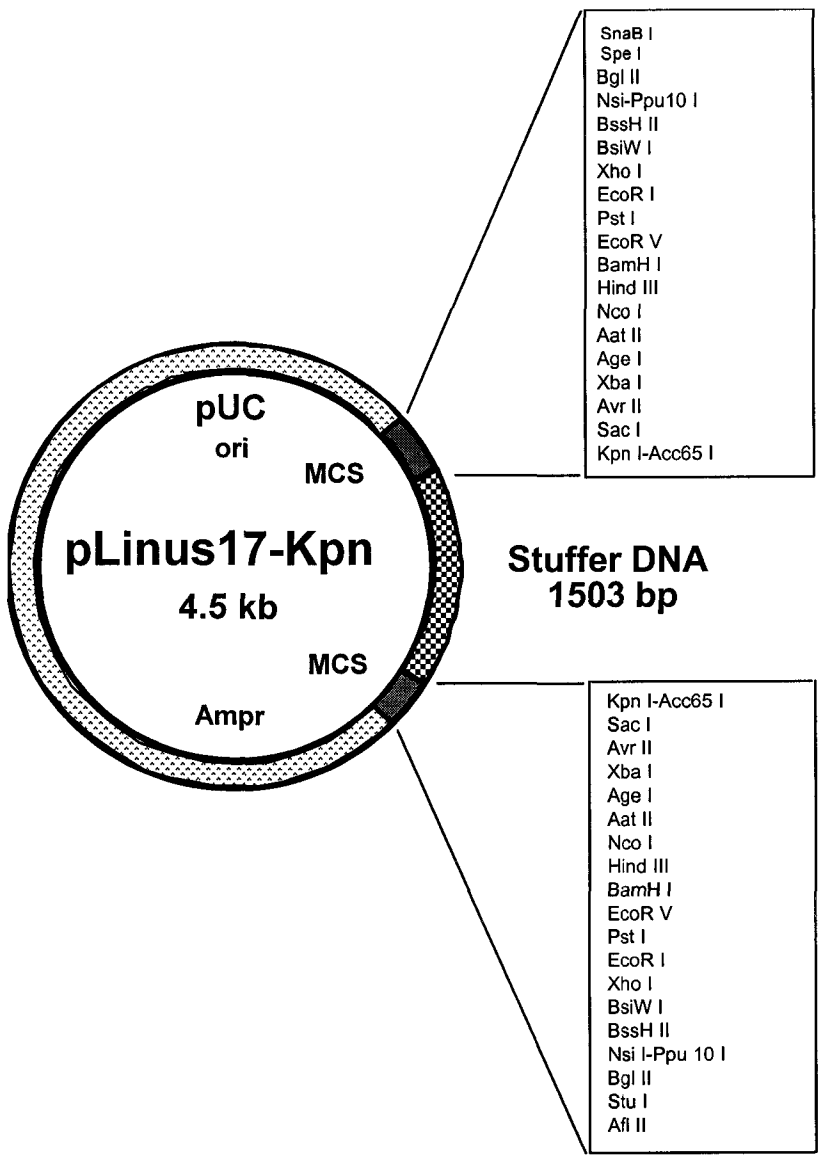


Figure 2A. Diagrammatic representation of the pLinus17-Kpn cloning vector. *pLinus17-Kpn* was derived by ligating the 2250-bp AlwN I-Kpn I fragment of Litmus29 with the 695-bp AlwN I-Kpn I fragment of Litmus 28. The stuffer DNA was a 1503-bp Kpn I fragment derived from Lambda DNA. MCS: multiple cloning sites.

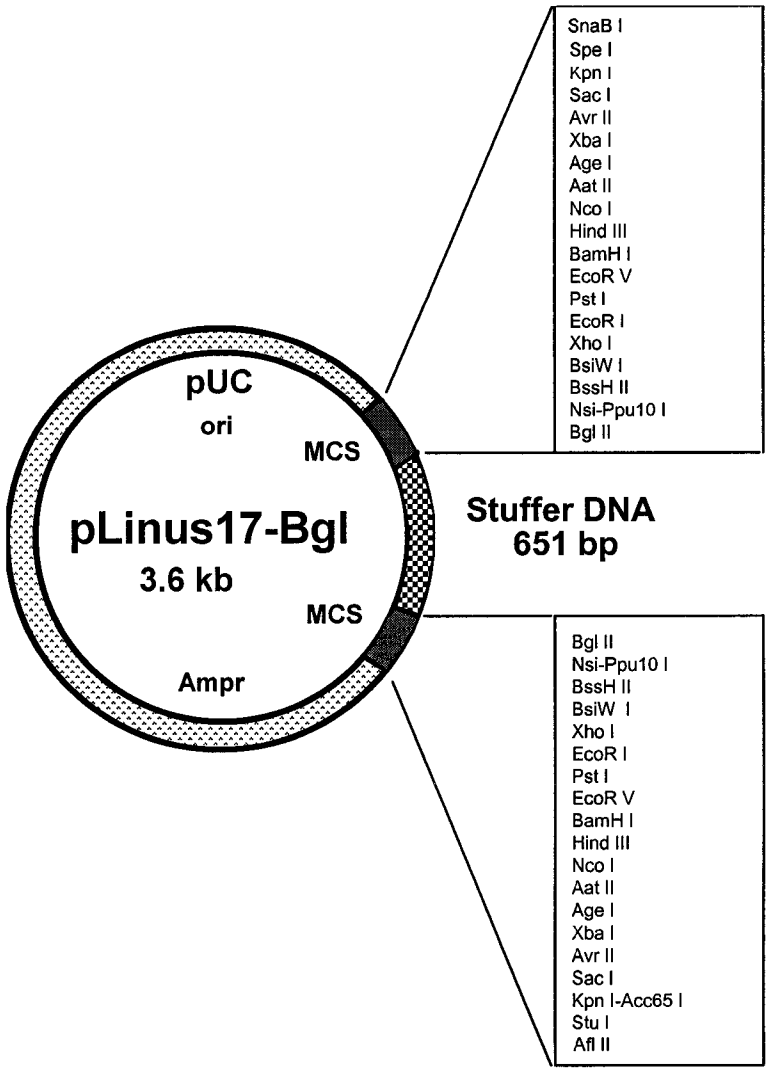


Figure 2B. Diagrammatic representation of the pLinus17-Bgl cloning vector. *pLinus17-Bgl* was derived by ligating the 2250-bp AlwN I-Bgl II fragment of Litmus 28 with the 695-bp AlwN I-Bgl II fragment of Litmus 29. The stuffer DNA was a 651-bp Bgl II fragment derived from Lambda DNA. MCS: multiple cloning sites.

subsequent handling of DNA pieces much easier and faster. The stuffer piece helps stabilize the plasmid structure by preventing the potential formation of secondary DNA structures between the two inverted repeated sequences. It also provides sufficient space between the two MCS for double digestions to work efficiently and allows verification that the digestion is complete by gel electrophoresis.

Analysis of the end products

To analyze the final products, the pLinus16-Apa and pLinus16-Sal were digested with various restriction enzymes and separated on a 1% agarose gel. The results showed that the 780-bp Apa I EGFP fragment from pcDNA3.1-EGFP was adapted for the Apa I, Hind III, BamH, Pst I, EcoR I, Nhe I and Sal I sites by using the pLinus16-Apa vector. Similarly, the 499-bp Sal I fragment from the Lambda DNA was adapted for these sites with the pLinus16-Sal vector (Figure 3A).

The pLinus17-Bgl and pLinus17-Kpn were also digested with various restriction enzymes and separated using a 1% agarose gel. The results showed that the 651-bp Bgl II fragment from the Lambda DNA was adapted for the Bgl II, Xho I, EcoR I, BamH I, Hind III, Xba I and Kpn I sites using the pLinus17-Bgl vector. Similarly, the 1503-bp Kpn I fragment from the Lambda DNA was adapted for these sites with the pLinus17-Kpn vector (Figure 3B).

Figure 3A

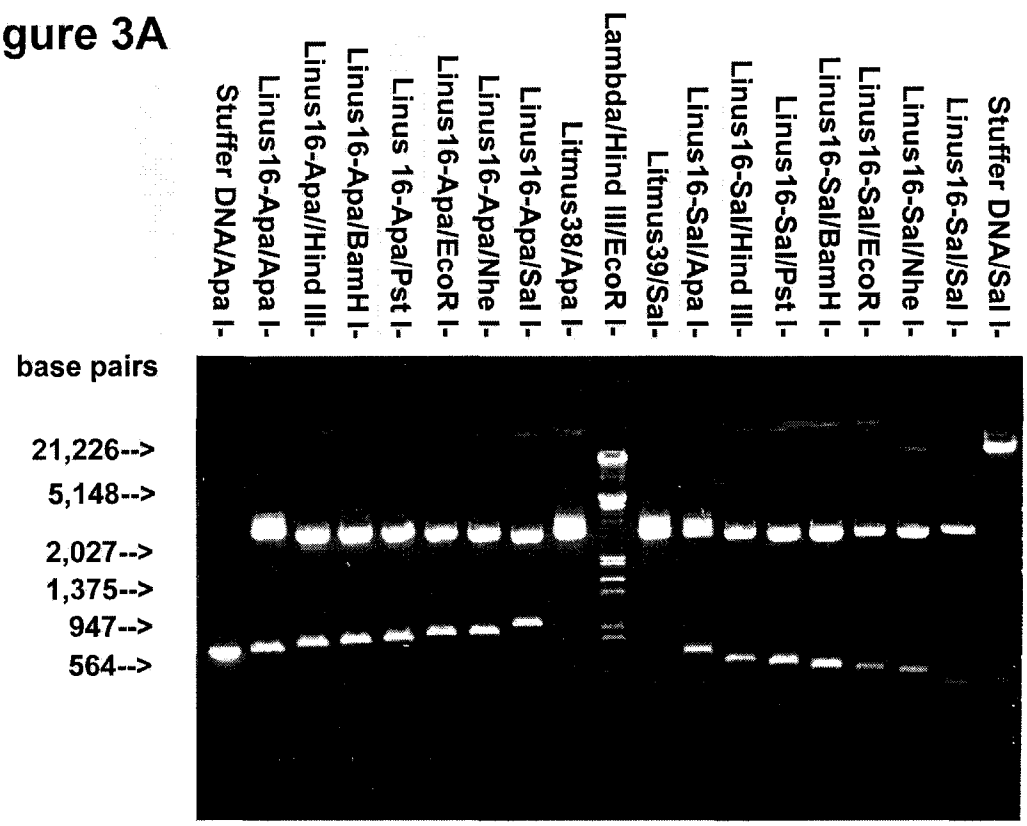


Figure 3A. Apa I- and Sal I-digested stuffer DNA fragments adapted for various sites using the pLinus16 vectors. pLinus16-Apa and pLinus16-Sal were digested with various restriction enzymes and separated on a 1% agarose gel. The sizes of the DNA fragments are indicated.

Figure 3B

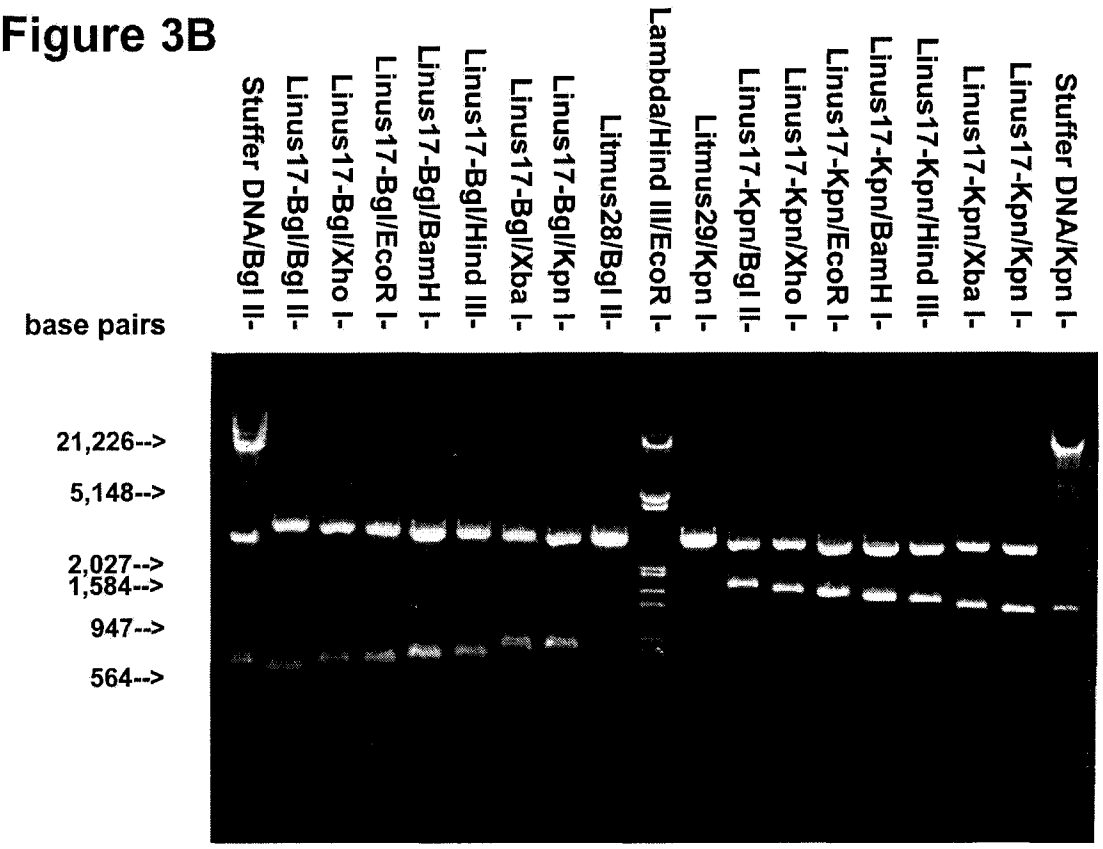


Figure 3B. Bgl II- and Kpn I-digested stuffer DNA fragments adapted for various sites using the pLinus17 vectors. pLinus17-Bgl and pLinus17-Kpn were digested with various restriction enzymes and separated on a 1% agarose gel. The sizes of the DNA fragments are indicated.

CHAPTER 3

DEVELOPMENT OF NEW HIGH GENE EXPRESSION PLASMIDS FOR IMPROVED EFFICACY IN CANCER GENE THERAPY

3.1 MATERIALS AND METHODS

Mice

Six- to eight-week old female C57BL/6, H-2^b mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed at the University of Arizona Animal Facilities in accordance with the principles of animal care (NIH publication No. 85-23, revised 1985)

Cell lines

Human lung cancer (A549) cells, human breast cancer (MCF-7) cells, mouse melanoma (B16) cells and mouse Lewis Lung carcinoma (LL/2) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproducts, Calabasas, CA), penicillin-streptomycin and gentamycin (Gibco-BRL, Rockville, MD). Cells were grown in an atmosphere of 5% CO_2 at 37°C and subcultured every 2-3 days using 0.05% trypsin.

Construction of the pCMV-IL-2 plasmid

The human IL-2 gene (gift from Dr. Evan Hersh, University of Arizona) was adapted for the EcoR I site of pCI-neo (Promega) using the Sac-KiSS-Lambda vector (86). Briefly, a 0.5 Kb BamH I-Pst I DNA fragment containing the IL-2 gene was inserted into Sac-KiSS-Lambda following a complete digestion with BamH I and a partial digestion with Pst I to create the plasmid, pSac-KiSS-IL-2. The IL-2 gene was then excised from pSac-KiSS-IL-2 as an EcoR I fragment and inserted into the EcoR I site of pCI-neo to generate the pCMV-IL-2 plasmid.

Construction of the pHi-2-IL-2 plasmid

The pHi-2-IL-2 plasmid was created by inserting the Tat gene into pHi2-IL-2-neo-C (40). Briefly, the TAT gene was excised from pTAT (40) with Xba I and ligated with Xba I digested Kpn-KiSS-Lambda (86) to create Kpn-KiSS-TAT. The TAT gene was then cut back out with Not I and inserted into the Not I site downstream of the CMV promoter in pHi2-IL-2-neo-C (40).

Construction of the pHi-1-IL-2 plasmid

The pHi-1-IL2 plasmid was created by inserting the Not I fragment from Kpn-KiSS-TAT into the Not I site of pHi-1-IL2-neo-C (40). The HIV 1 promoter was excised with Hind III from pGL2-HIV1 (40) and replaced the HIV 2 promoter in pHi-IL-2 to form pHi-1-IL-2.

Construction of the pCMV-EGFP plasmid

The enhanced Green Fluorescent Protein (EGFP) gene was excised from pcDNA3.1-EGFP (See Appendix B) and inserted into the EcoR I site of pCI-neo to generate the pCMV-EGFP plasmid. The pcDNA3.1-EGFP was constructed by insertion of a 758-bp Kpn I/Not I EGFP fragment from pEGFP-1 (BD Biosciences Clontech, Palo Alto, CA) into the Kpn I/Not I sites of pcDNA3.1 (Invitrogen, Carlsbad, CA).

Construction of the pHi-2-EGFP plasmid

The enhanced Green Fluorescent Protein (EGFP) gene was excised from pcDNA3.1-EGFP and inserted into the Nhe I / Not I sites of pHi-2-IL-2 by replacing the IL-2 fragment to generate the pHi-2-EGFP plasmid.

Construction of the pHi-1-EGFP plasmid

The enhanced Green Fluorescent Protein (EGFP) gene was excised from pcDNA3.1-EGFP and inserted into the Nhe I / Not I sites of pHi-1-IL-2 by replacing the IL-2 fragment to generate the pHi-1-EGFP plasmid.

In vitro transfections using the DMIRE-C reagent

Cells were seeded at $2x10^5$ cells/well in a six well tissue culture plate in 2ml complete RPMI 1640 medium and incubated at 37°C until they were 40–60% confluent. The DMRIE-C reagent (Invitrogen, Carlsbad, CA) was used in all

transfections. The plasmids used in transient transfections were pHi-1-IL-2, pHi-2-IL-2, pCMV-IL-2-IL-2, pHi-1-EGFP, pHi-2-EGFP and pCMV-EGFP. DNA/DMRIE-C ratios used for transfections were either 1:2 or 1:6 for all cells. For each well, 1μg of test plasmid vector DNA was suspended in 500μl OPTI Reduced Serum Medium (Gibco-BRL, Rockville, MD). 2μl or 6μl DMRIE-C lipid was suspended in 500μl OPTI medium. The DNA and DMRIE-C solutions were mixed together and incubated at room temperature for 30 minutes. Cells were then washed with OPTI medium and incubated with 1ml DNA/lipid complex mixture at 37°C. The mixtures were replaced by 2ml RPMI culture medium 4h later. Supernatants from samples transfected with pHi-1-IL-2, pHi-2-IL-2 and pCMV-IL-2 vectors were harvested 48h post-transfection and stored at –80°C until analyzed. For samples transfected with the pHi-1-EGFP, pHi-2-EGFP and pCMV-EGFP vectors, 48h post-transfection, the medium from each sample was removed, the cells were trypsinized, and washed with RPMI medium. Cell pellets were then resuspended and fixed in 500μl 1% paraformaldehyde for FACS analysis (See Apendix C). All transfections were done in triplicate.

Establishment of stably transfected tumor cell clones

Using a DNA/DMRIE-C ratio of 1μg/6μl, the pHi-2-IL-2 and pCMV-IL-2 vectors were transfected into LL/2 tumor cells using the same protocol described above. The cells were seeded into 96-well tissue culture plates (Falcon, Becton Dickinson labware: Lincoln Park, NJ) at 500-800 cells/well 48h after

transcfection. Vector carrying clones were selected in 200μl of G418 (Gibco-BRL, Rockville, MD)-containing medium at the concentration of 1μg/ml. After 2 weeks approximately 20 resistant colonies from each transfected group were selected by serial dilution and expanded for future experiments. All isolated clones were screened for IL-2 expression and then frozen at –80°C for future study.

IL-2 ELISA

IL-2 secretion into cell supernatants was measured using a human IL-2 ELISA kit (Pharmingen, San Diego, CA) using protocols supplied by the manufacturer. Briefly, a 96-well microtiter plate was coated with capture monoclonal antibodies specific to human IL-2. 100μl of supernatant from each triplicate samples was added to wells, either undiluted or after dilution, and incubated for 2 hours at room temperature. The wells were then rinsed 5-7 times with washing buffer. Detection antibody conjugated to horseradish peroxidase was then added. The plate was incubated for an additional 1h at room temperature, the wells were washed as before, and 100μl of substrate solution was added to each well. After 30 min at room temperature, the reaction was stopped by the addition of 2N H_2SO_4 and the plate was read on a microplate reader at an absorbance of 450nm. A standard curve was plotted using recombinant IL-2 provided in the assay kit and the IL-2 concentrations were determined by interpolation from the standard curve. Results were calculated as pg/ml of IL-2.

Fluorescence activated cell sorting (FACS) analysis of EGFP expressing cells

At 48h post-transfection, the transfected cells were pelleted and fixed in 1% paraformaldehyde for 5 min. All samples were analyzed using a Becton Dickinson FACScan flow cytometer equipped with a 15-mW air-cooled 488-nm argon ion laser. The green fluorescence (i.e., GFP) was detected using a 530/30-nm band pass filter. FACS data were acquired and analyzed on a Hewlett-Packard (San Diego, CA) 340 computer. Log green fluorescence histograms were illustrated using the Lysys II software package (Becton Dickinson, San Jose, CA).

Determination of in vitro proliferation of stable LL/2 tumor cell clones

To select clones with similar growth rates for in vivo injection, the in vitro growth characteristics of wild type LL/2 tumor cells and the different plasmid-modified LL/2 tumor clones was assessed. Cells were seeded in triplicate at $2x10^5$ cells per 2ml/well in 6-well tissue culture plates (Falcon, Becton Dickinson labware: Lincoln Park, NJ) and incubated at 37°C for 48h. Cells were then collected from triplicate plates 48h later and counted by trypan blue dye exclusion (see APPENDIX D for Viability Staining Protocol).

Determination of tumorigenicity of LL/2 stable tumor cell clones

The tumorigenicity of various stably transfected LL/2 clones and parental LL/2

tumor cells was determined by monitoring primary tumor growth rate in syngeneic mice. Primary tumors were generated by subcutaneous injection of 5 x 10^5 cells in 100μl PBS in the hind flank of each C57BL/6 mouse. Tumors were measured in two perpendicular dimensions every other day for 4 weeks using vernier calipers (VWR Scientific products, Willard, OH). Tumor volume was calculated using the formula $v=(l)(w^2)/2$, where v = volume (mm^3), l = long diameter, and w = short diameter.

Determination of serum IL-2 levels in mice

The IL-2 levels in sera of mice injected with pHi-2-IL-2, pCMV-IL-2, pHi-2-MCS, pCMV-MCS vector-modified stable clones and the parental tumor cells, were measured and compared. Blood samples (200μl / mouse) were collected from the retro-orbital sinus starting at day 1, 3, 7,10 after tumor cell injection. The collected blood samples were first let to sit at room temperature for 30 minutes, then centrifuged at 4000 rpm (2500*g*) for 10 minutes and harvest the supernatants. The collected sera were frozen at –80°C and analyzed for IL-2 expressions by IL-2 ELISA assay.

Statistical analysis

Results are given as the mean ± standard error of the mean (SEM). Differences between groups in in vitro tests and differences in tumor diameter in in vivo experiments were analyzed for significance by student's t-test (two-tailed). Statistical significance was set at $p < 0.05$.

3.2 RESULTS

Design and construction of high expression plasmid vectors pHi-1/2-MCS using a transcriptional amplification strategy.

To achieve optimal efficacy in gene therapy, high therapeutic dosages of transgenes must often be reached. In the case of plasmid vectors, enhancement of expression levels by using optimized transcriptional regulatory elements should be a practical way to compensate for its low efficiency in gene delivery. In this regard, two new high expression plasmid vectors pHi-1-MCS (Figure 4) and pHi-2-MCS (Figure 5) were designed and constructed in which a transcriptional amplification strategy was incorporated. Both of the pHi-1/2-MCS amplifier vectors consist of three independent transcriptional units instead of using a viral internal ribosomal entry site (IRES), which usually results in much lower co-expression of the gene put behind it (87,88). In the pHi-1-MCS amplifier vector, the first transcriptional unit contains a gene encoding the transcriptional activator, Tat, driven by a constitutively strong CMV promoter. The second transcriptional unit contains an HIV1 LTR promoter driving the gene of interest. The pHi-2-MCS amplifier vector was identical to the pHi-1-MCS vector, except that the second promoter in this vector was the HIV2 LTR. The rationale of incorporating an amplifier strategy using a multi-gene expression cassette is that a transcriptional feedback positive loop will be established in a single vector. Theoretically, the

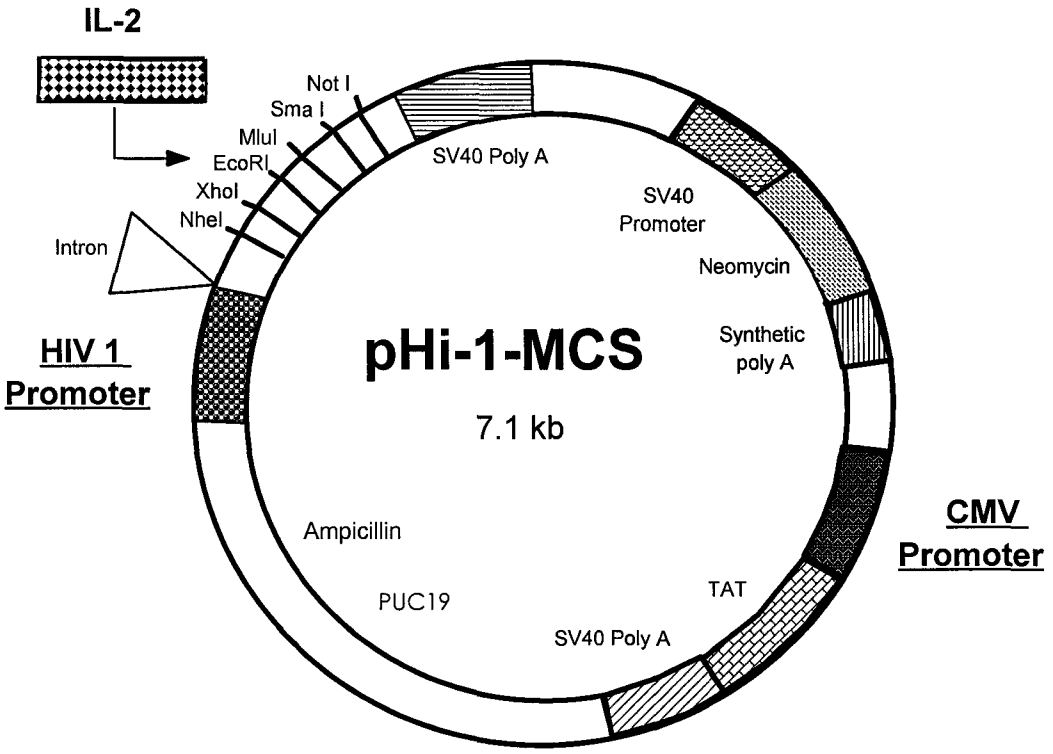


Figure 4. Diagrammatic representation of the pHi-1-IL-2 plasmid. The first gene encodes a transcriptional activator (Tat) driven by a constitutive CMV promoter. The second promoter (HIV1 LTR) drives the human IL-2 cytokine gene. MCS: multiple cloning sites

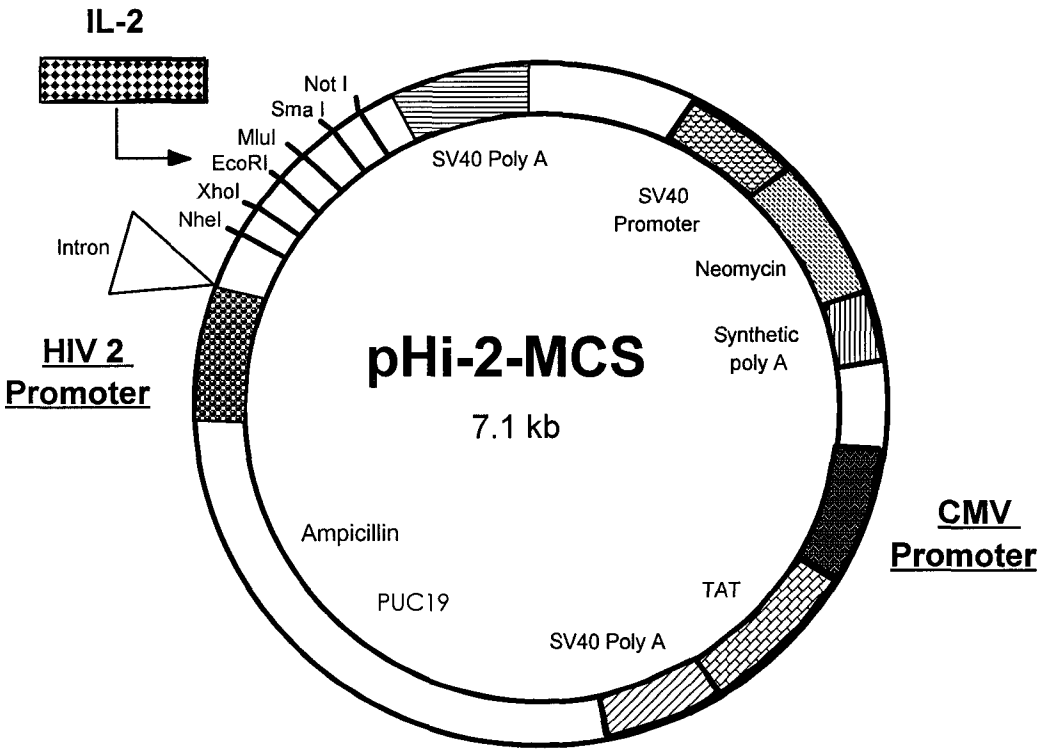


Figure 5. Diagrammatic representation of the pHi-2-IL-2 plasmid. The first gene encodes a transcriptional activator (Tat) driven by a constitutive CMV promoter. The second promoter (HIV2 LTR) drives the human IL-2 cytokine gene. MCS: multiple cloning sites

HIV1/2 LTR promoter activities should be enhanced by the amplifier (Tat) expressed in the same construct. This enhancement consequently could lead to an increased expression level of the second gene. The third transcriptional unit contains the neomycin/kanamycin resistance gene under the control of the bacterial Ampicillin gene promoter and the SV40 promoter to allow for selection in both mammalian cells and bacteria. The transgene of interest can be directly cloned into the pHi-1/2-MCS vectors at the compatible restriction sites present in its multiple cloning site (MCS) downstream of the HIV1/2 LTR promoter. The large piece of MCS present in pHi-1/2 amplifier vectors contains six commonly used restriction sites that allow convenient insertion of the gene(s) into these vectors. In this study, the human IL-2 cytokine gene was used as a reporter gene and as a therapeutic gene.

The CMV promoter is considered one of the strongest promoters available and has already been widely used in gene therapy research. Using the same plasmid backbone, a control plasmid, pCMV-IL-2, was constructed in which the IL-2 gene was placed under the direct control of the strong constitutive CMV promoter (Figure 6). The IL-2 levels from the pHi-1/2 amplifier vectors would be compared to that obtained from the pCMV-IL-2 vector. Also, the efficiency of Tat transactivation on the HIV1 LTR and HIV2 LTR can be tested and compared by measuring the IL-2 production from the pHi-1-IL-2 and pHi-2-IL-2 amplifier vectors.

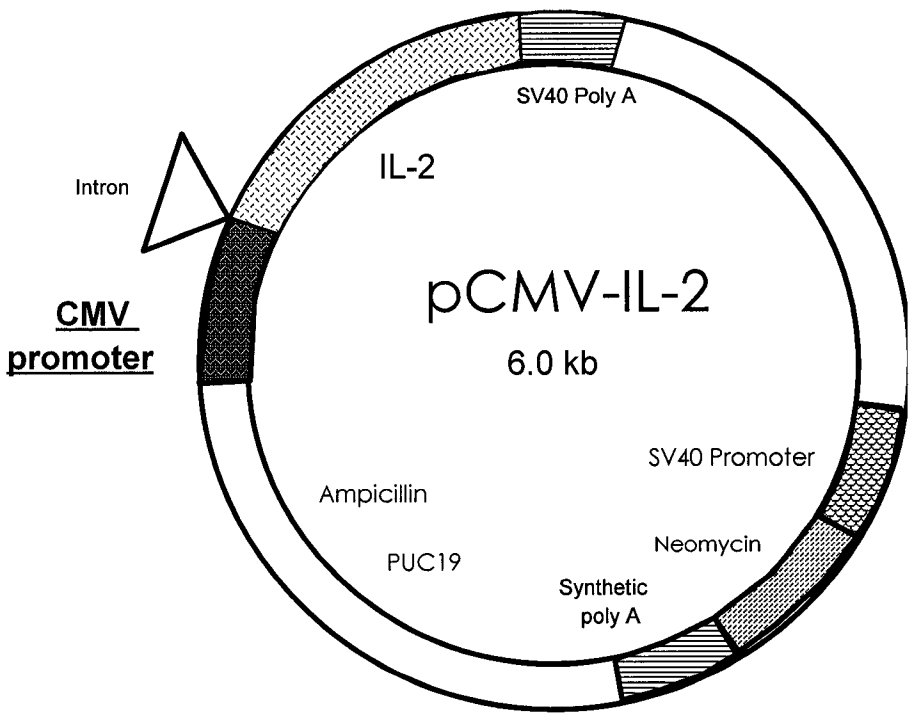


Figure 6. Diagrammatic representation of the pCMV-IL-2 plasmid. The constitutively active CMV promoter directly drives the human cytokine IL-2 gene.

Determination of IL-2 production from pHi-1/2 amplifier vectors transfected into human A549 and MCF-7 tumor cells.

Cationic lipids are frequently used in plasmid-based gene delivery (24,89-91). Thus, the DMRIE-C reagent was used for all transfections described in this study. To determine IL-2 production from pHi-1-IL-2 and pHi-2-IL-2, these two vectors were transfected into human lung cancer (A549) cells. The pCMV-IL-2 vector was used as a control. For these transient transfections, a DNA/DMRIE-C ratio of 1 μg/2μl and 1μg/6μl was used. All transfections were done in triplicate. The supernatant from each sample was collected 48h post-transfection and measured for IL-2 production by ELISA. In A549 cells, using a DNA/DMRIE-C ratio of 1μg/2μl, more IL-2 production was detected from samples transfected with each of the three plasmids, as compared to using a DNA/DMRIE-C ratio of 1μg/6μl (Figure 7). Under the latter transfection conditions, increased cell death (approximately 46%) was observed in all transfected samples (Table 1). However, under either transfection condition, transient IL-2 production by pHi-1-IL-2 and pHi-2-IL-2 was significantly higher than that obtained from the pCMV-IL-2 control vector (Figure 7). The IL-2 production by cells transfected with the pHi-2-IL-2 (21006 ± 2120 pg/ml) and pHi-1-IL-2 (17785 ± 1710 pg/ml) vectors were 7.8- and 6.5-fold higher, respectively, than that obtained from cells transfected with the control pCMV-IL-2 vector (2714 ± 870 pg/ml) using the optimal DNA/DMRIE-C ratio of 1μg/2μl. Identical transfections using these plasmids were performed with the human breast cancer cell line, MCF-7. A similar IL-2

production pattern was observed in MCF-7 cells that were transfected with the pHi-1/2-IL-2 amplifier vectors. Using a DNA/DMRIE-C ratio of 1μg/2μl yielded higher IL-2 production with less cell death in each transfected sample as compared to using 6ul of DMRIE-C (Table 2). Under either transfection condition, the transient IL-2 production obtained from pHi-1-IL-2 and pHi-2-IL-2 was significantly higher than that observed with the pCMV-IL-2 vector (Figure 8). The IL-2 production from the pHi-2-IL-2 (27898 ± 2480 pg/ml) and pHi-1-IL-2 (20550 ± 2100 pg/ml) vectors were 12- and 9-fold higher than that obtained from the control pCMV-IL-2 vector (2266 ± 462 pg/ml) using the optimal transfection condition of DNA/DMRIE-C at a ratio of 1μg/2μl. In both A549 and MCF-7 cell lines, the pHi-2-IL-2 plasmid produced more IL-2 than the pHi-1-IL-2 plasmid (Figures 7 and 8).

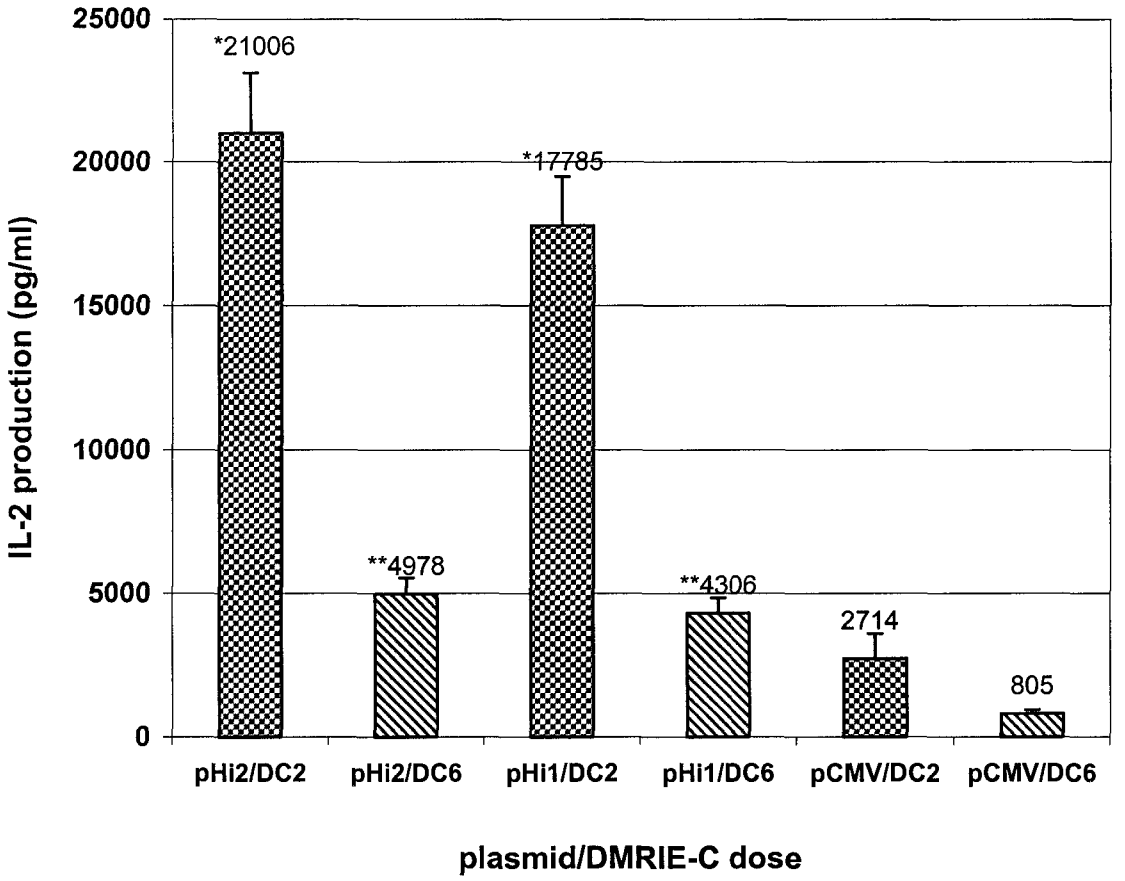


Figure 7. IL-2 production in A549 cells using different DNA/DMRIE-C doses. The A549 cells were transfected with DNA/DMRIE-C either at the ratio of 1μg/2μl or 1μg/6μl. Supernatants were harvested 48h post-transfection. IL-2 production was measured by ELISA. Each bar represents for the IL-2 production. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments. *p < 0.005 when compared to the IL-2 level obtained from the pCMV-IL-2 control vector using DNA/DMRIE-C at 1μg/2μl. **p < 0.005 when compared to the IL-2 level obtained from the pCMV-IL-2 control vector using DNA/DMRIE-C at 1μg/6μl. pHi1: pHi-1-IL-2 vector; pHi2: pHi-2-IL-2; pCMV: pCMV-IL-2; DC2: 2μl DMRIE-C; and DC6: 6μl DMRIE-C

plasmid DNA/DMRIE-C (ratio)	cell line	cell number ± SEM	cell viability (%)
control	A549	$5.2 \pm 0.6 \times 10^5$	100
pHi-1-IL-2 (1ug/2ul) (1ug/6ul)	A549	$4.0 \pm 0.8 \times 10^5$ $2.4 \pm 0.75 \times 10^5$	77 46
pHi-2-IL-2 (1ug/2ul) (1ug/6ul)	A549	$3.8 \pm 0.65 \times 10^5$ $2.5 \pm 0.75 \times 10^5$	73 48
pCMV-IL-2 (1ug/2ul) (1ug/6ul)	A549	$4.0 \pm 0.75 \times 10^5$ $2.4 \pm 0.8 \times 10^5$	77 46
pHi-1-EGFP (1ug/2ul) (1ug/6ul)	A549	$4.2 \pm 0.6 \times 10^5$ $2.35 \pm 0.75 \times 10^5$	80 45
pHi-2-EGFP (1ug/2ul) (1ug/6ul)	A549	$3.9 \pm 0.75 \times 10^5$ $2.5 \pm 0.55 \times 10^5$	75 48
pCMV-EGFP (1ug/2ul) (1ug/6ul)	A549	$4.25 \pm 0.65 \times 10^5$ $2.3 \pm 0.85 \times 10^5$	81 45

Table 1. Cell viability in A549 cells transfected with DNA/DMRIE-C at different ratio. A549 cells were seeded at 2.0×10^5 cells/well in the 6-well plates. The pHi-1-IL-2, pHi-2-IL-2, pCMV-IL-2, pHi-1-EGFP, pHi-2-EGFP and pCMV-EGFP plasmids were transfected into the cells, respectively, 24h later. The non-transfected tumor cells were used as control. All cells were harvested 48h post-transfection and counted for cell viability. Each cell number represents the mean ± SEM of triplicate samples from one of the three independent experiments.

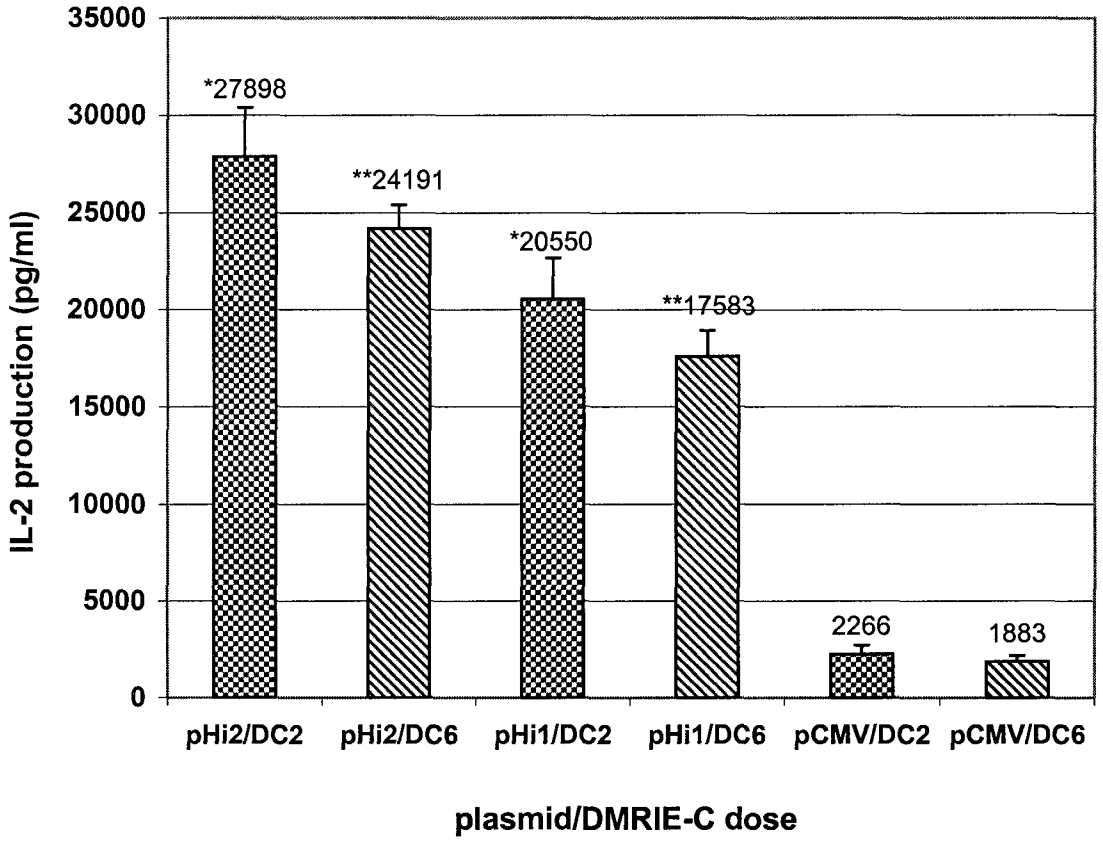


Figure 8. IL-2 production in MCF-7 cells using different DNA/DMRIE-C doses. The MCF-7 cells were transfected with DNA/DMRIE-C either at the ratio of 1μg/2μl or 1μg/6μl. Supernatants were harvested 48h post-transfection. IL-2 production was measured by ELISA. Each bar represents for the IL-2 production. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments. *$p < 0.005$ when compared to the IL-2 level obtained from the pCMV-IL-2 control vector using DNA/DMRIE-C at 1μg/2μl. **$p < 0.005$ when compared to the IL-2 level obtained from the pCMV-IL-2 control vector using DNA/DMRIE-C at 1μg/6μl. pHi1: pHi-1-IL-2 vector; pHi2: pHi-2-IL-2; pCMV: pCMV-IL-2; DC2: 2μl DMRIE-C and DC6: 6μl DMRIE-C

plasmid DNA/DMRIE-C (ratio)	cell line	cell number ± SEM	cell viability (%)
control	MCF-7	$5.0 \pm 0.5 \times 10^5$	100
pHi-1-IL-2 (1ug/2ul) (1ug/6ul)	MCF-7	 $3.8 \pm 0.45 \times 10^5$ $2.7 \pm 0.65 \times 10^5$	 76 54
pHi-2-IL-2 (1ug/2ul) (1ug/6ul)	MCF-7	 $3.9 \pm 0.5 \times 10^5$ $2.9 \pm 0.65 \times 10^5$	 78 58
pCMV-IL-2 (1ug/2ul) (1ug/6ul)	MCF-7	 $3.65 \pm 0.65 \times 10^5$ $2.75 \pm 0.75 \times 10^5$	 73 55
pHi-1-EGFP (1ug/2ul) (1ug/6ul)	MCF-7	 $3.75 \pm 0.45 \times 10^5$ $2.8 \pm 0.5 \times 10^5$	 75 56
pHi-2-EGFP (1ug/2ul) (1ug/6ul)	MCF-7	 $3.8 \pm 0.7 \times 10^5$ $2.9 \pm 0.45 \times 10^5$	 76 58
pCMV-EGFP (1ug/2ul) (1ug/6ul)	MCF-7	 $3.85 \pm 0.5 \times 10^5$ $2.8 \pm 0.65 \times 10^5$	 77 56

Table 2. Cell viability in MCF-7 cells transfected with DNA/DMRIE-C at different ratio. MCF-7 cells were seeded at 2.0×10^5 cells/well in the 6-well plates. The pHi-1-IL-2, pHi-2-IL-2, pCMV-IL-2, pHi-1-EGFP, pHi-2-EGFP and pCMV-EGFP plasmids were transfected into the cells, respectively, 24h later. The non-transfected tumor cells were used as control. All cells were harvested 48h post-transfection and counted for cell viability. Each cell number represents the mean ± SEM of triplicate samples from one of the three independent experiments.

Determination of transfection efficiency of pHi-1/2 amplifier vectors in human A549 and MCF-7 tumor cells.

In transient transfections, it was shown that both pHi-1-IL-2 and pHi-2-IL-2 amplifier vectors produced significantly higher levels of IL-2 than the pCMV-IL-2 vector. The next step was to determine whether the higher IL-2 production from the pHi-1/2 amplifier vectors resulted from an increased transfection efficiency. Using EGFP as a reporter gene, the pHi-1-EGFP (Figure 9) and pHi-2-EGFP (Figure 10) vectors were transfected into A549 cells. The pCMV-EGFP (Figure 11) vector was used as a control. For each tested plasmid, DNA/DMRIE-C ratios of 1μg/2μl and 1μg/6μl were used under the same transfection conditions described above. At 48h post-transfection, the transfected samples were fixed in 500μl 1% paraformaldehyde (See Appendix C). The transfection efficiency of each plasmid was determined by measuring the percentage of EGFP-expressing cells by FACS analysis. Figure 12 showed, in A549 cells, using a DNA/DMRIE-C ratio of 1μg/6μl, the percentage of EGFP- expressing cells from pHi-2-EGFp, pHi-1-EGFP and pCMV-EGFP were 68 ± 6%, 73 ±7.5 % and 71 ± 7.8%, respectively. When using DNA/DMRIE-C a ratio of 1μg/2μl, the percentage of EGFP-expressing cells from pHi-2-EGFP, pHi-1-EGFP and pCMV-EGFP were 54 ± 5%, 57 ± 6.5 % and 60 ± 7.3%, respectively. However, more cell death was observed when using 6μl DMRIE-C (Table 1). Under any transfection condition, 48h post-transfection the percentage of EGFP-expressing cells transfected with pHi-1/2 amplifier vectors was comparable to those cells transfected with the pCMV-

EGFP control vector (Figure 12). When the same plasmids were transfected into the MCF-7 cell line, a similar pattern of expression was observed 48h post-transfection. Using a DNA/DMRIE-C ratio of 1μg/6μl resulted in a higher percentage of EGFP-expressing cells (pHi-2-EGFP: 60 ± 6.2%, pHi-1-EGFP: 62 ± 6.5% and pCMV-EGFP: 67 ± 7.8%) but with more cell death observed (Table 2) compared with the lower DMRIE-C dose (pHi-2-EGFP: 40 ± 5 %, pHi-1-EGFP: 47 ± 8 % and pCMV-EGFP: 50 ± 6%). Under either transfection condition, the percentage of EGFP-expressing cells from the pHi-1/2-EGFP vectors was comparable to that of the pCMV-EGFP vector (Figure 13).

In both of the human cell lines, a higher transfection efficiency could be achieved by using a higher DMRIE-C dose. However, more cell death was observed because of the toxicity of the higher dose of DMRIE-C. This observation might explain why lower IL-2 production was detected in all samples transfected using the higher dose of DMRIE-C (Figures 7 and 8). Under either transfection condition, the pHi-1/2 amplifier vectors achieved a similar transfection efficiency as compared to the control pCMV vector. This finding indicated that the higher IL-2 levels obtained from the two amplifier vectors resulted from an enhanced transcriptional activity of the HIV1/2 LTR promoter when using the amplifier (Tat) strategy.

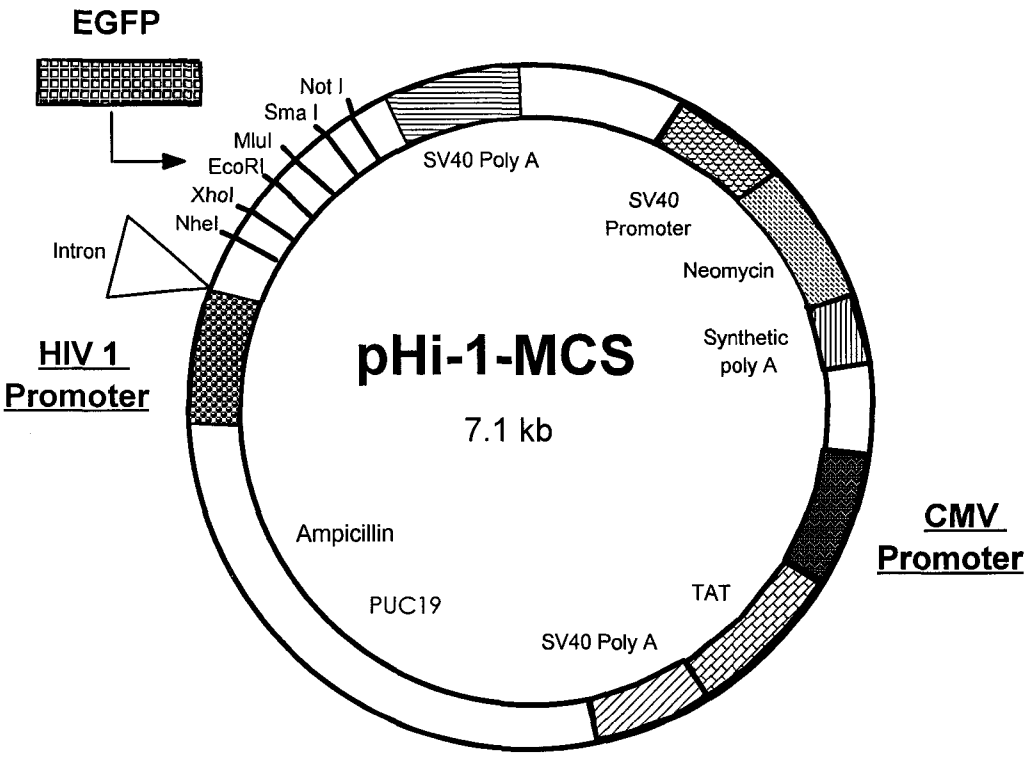


Figure 9. Diagrammatic representation of the pHi-1-EGFP plasmid. The first gene encodes a transcriptional activator (Tat) driven by a constitutive CMV promoter. The second promoter (HIV1 LTR) drives the EGFP gene. MCS: multiple cloning sites

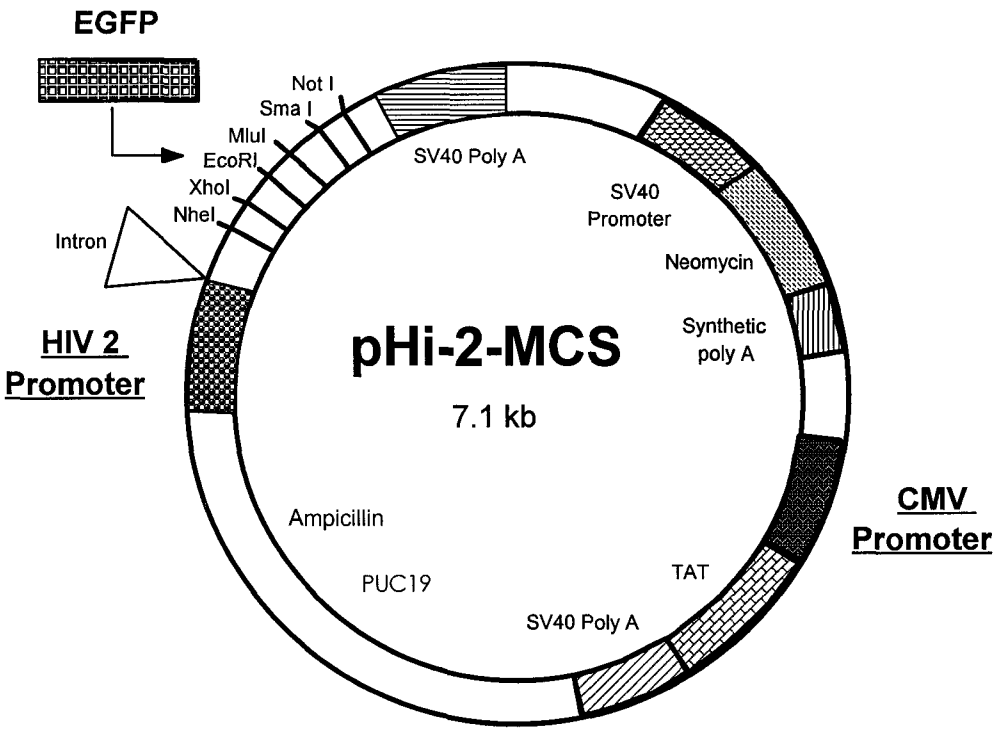


Figure 10. Diagrammatic representation of the pHi-2-EGFP plasmid. The first gene encodes a transcriptional activator (Tat) driven by a constitutive CMV promoter. The second promoter (HIV2 LTR) drives the EGFP gene. MCS: multiple cloning sites

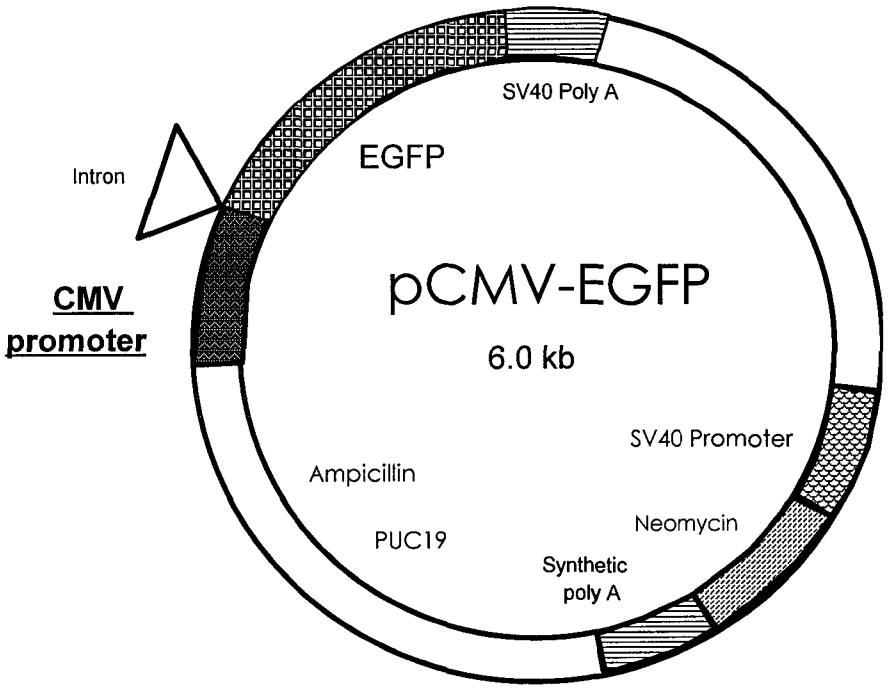


Figure 11. Diagrammatic representation of the pCMV-EGFP plasmid. The constitutively active CMV promoter directly drives the EGFP gene.

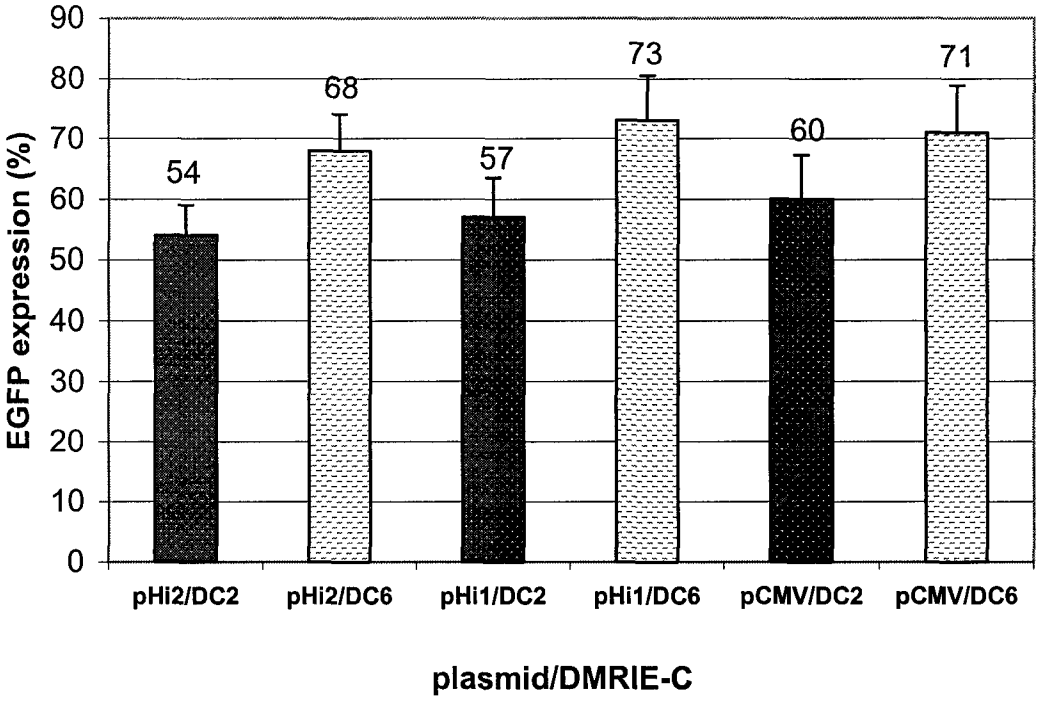


Figure 12. Percentage of EGFP-expressing A549 cells using different DNA/DMRIE-C ratios. The A549 cells were transfected with DNA/DMRIE-C either at a ratio of 1μg/2μl or 1μg/6μl. EGFP expressing cells were analyzed by FACS. Each bar represents the percentage of EGFP expressing cells at 48h post-transfection. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments. pHi1: pHi-1-EGFP vector; pHi2: pHi-2-EGFP; pCMV: pCMV-EGFP; DC2: 2μl DMRIE-C and DC6: 6μl DMRIE-C

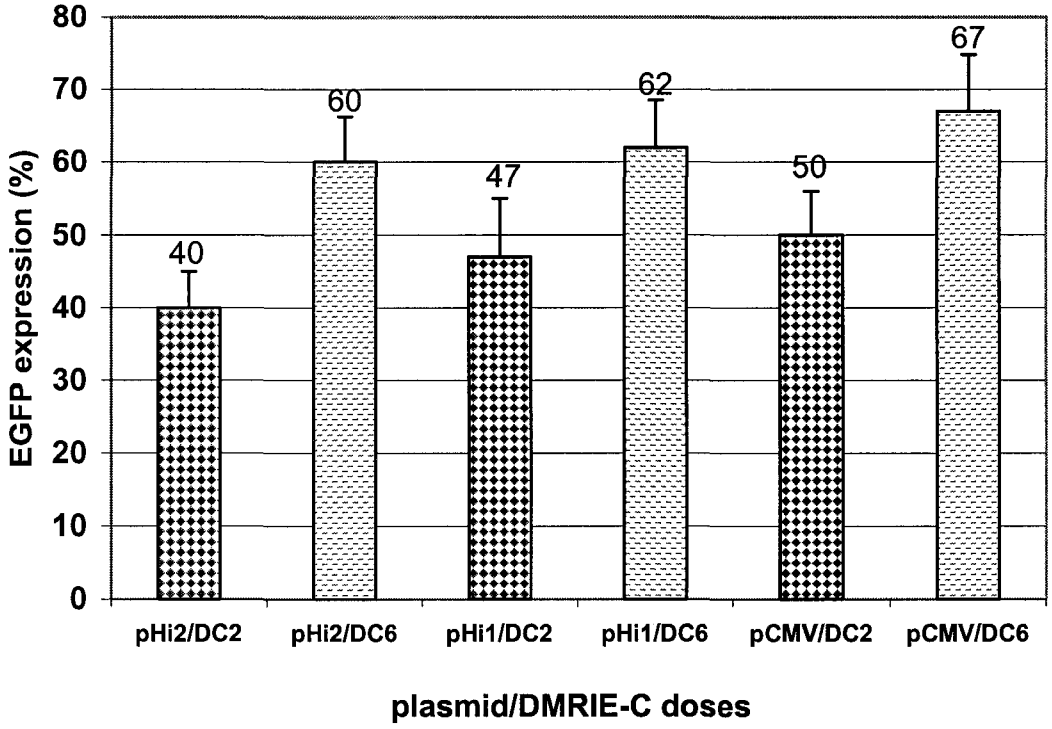


Figure 13. Percentage of EGFP-expressing MCF-7 cells using different DNA/DMRIE-C ratios. The MCF-7 cells were transfected with DNA/DMRIE-C either at the ratio of 1μg/2μl or 1μg/6μl. Cells were fixed at 48h post-transfection. EGFP expressing cells were analyzed by FACS. Each bar represents the percentage of EGFP expressing cells at 48h post-transfection. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments. pHi1: pHi-1-EGFP vector; pHi2: pHi-2-EGFP; pCMV: pCMV-EGFP; DC2: 2μl DMRIE-C and DC6: 6μl DMRIE-C

Determination of IL-2 production by the pHi-1/2 amplifier vectors in murine B16 and LL/2 tumor cells.

To determine the IL-2 production obtained from the pHi-1-IL-2 and pHi-2-IL-2 vectors in murine tumor cells, these two amplifier vectors were transfected into mouse melanoma (B16) and Lewis Lung carcinoma (LL/2) tumor cells. Again, the pCMV-IL-2 vector was used as a control. The same two DNA/DMRIE-C ratios were used in this study as before. In contrast to what was observed in the human A549 and MCF-7 cell lines, in mouse B16 and LL/2 cell lines, when using a DNA/DMRIE-C ratio of 1μg/6μl, higher IL-2 production was detected in the supernatants from all transfected samples as compared to using DNA/DMRIE-C at a ratio of 1μg/2μl (Figures 14 and 15). Using a DNA/DMRIE-C ratio of 1μg/6μl, IL-2 productions were 2938 ± 220pg/ml (pHi-2-IL-2), 1602 ± 130pg/ml (pHi-1-IL-2) and 1761 ± 189pg/ml (pCMV-IL-2) in B16 cells and 14350 ± 1380pg/ml (pHi-2-IL-2), 4571 ± 830pg/ml (pHi-1-IL-2) and 3721 ± 790pg/ml (pCMV-IL-2) in LL/2 cells. Using a DNA/DMRIE-C ratio of 1ug/2ul, IL-2 productions were 962 ± 80pg/ml (pHi-2-IL-2), 521 ± 110pg/ml (pHi-1-IL-2) and 750 ± 162pg/ml (pCMV-IL-2) in B16 cells and 198 ± 53pg/ml (pHi-2-IL-2), 65 ± 18pg/ml (pHi-1-IL-2) and 231 ± 53pg/ml (pCMV-IL-2) in LL/2 cells. Under either transfection condition, no significant cell death was observed in any of the transfected samples (Table 3 and 4). The IL-2 production from the pHi-1/2 amplifier vectors in either murine cell line was not higher than that obtained with the control pCMV-IL-2 vector when using DNA/DMRIE-C at a 1μg/2μl ratio. Only when using DNA/DMRIE-C at

a 1μg/6μl ratio did the pHi-2-IL-2 vector produce significantly more IL-2 than the pCMV-IL-2 control vector in B16 cells (2938 ± 220 pg/ml vs 1761 ± 189 pg/ml) and LL/2 cells (14305 ± 1380 pg/ml vs 3721 ± 790 pg/ml). The pHi-1-IL-2 vector produced comparable amounts of IL-2 as compared to the control pCMV-IL-2 vector. Under either transfection condition, the pHi-2-IL-2 vector produced more IL-2 than the pHi-1-IL-2 amplifier vector for both B16 and LL/2 cell lines (Figures 14 and 15).

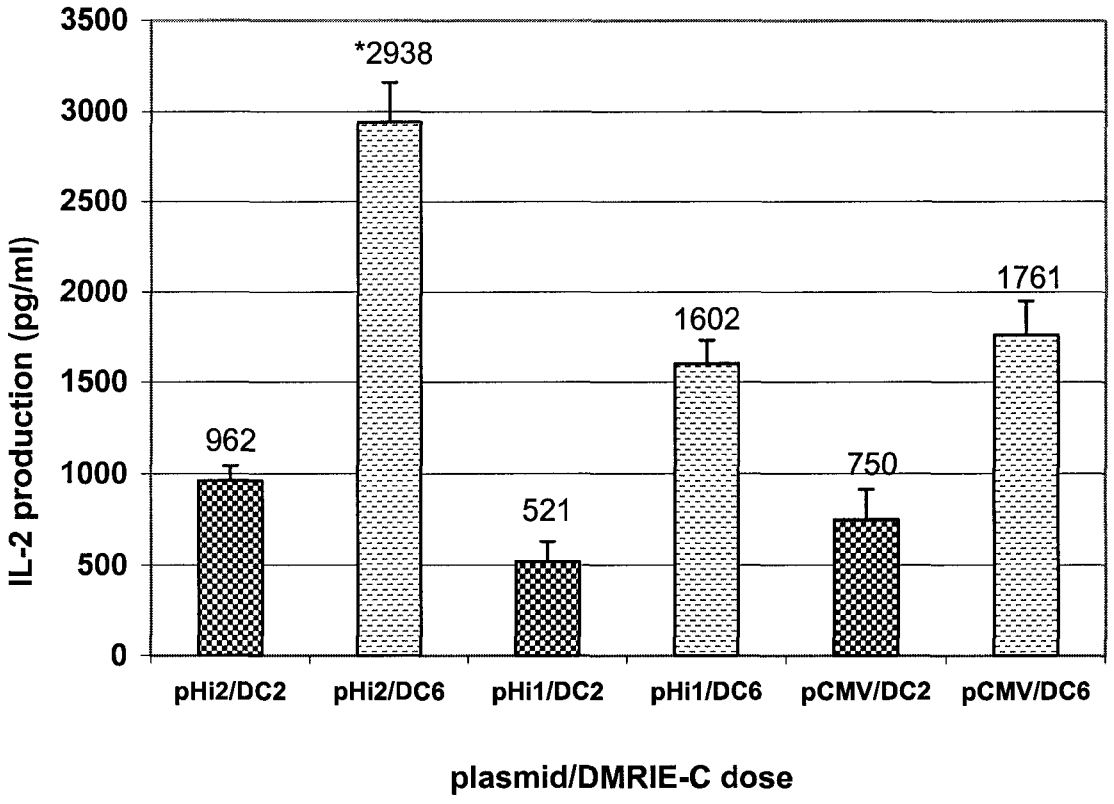


Figure 14. IL-2 production in B16 tumor cells using different DNA/DMRIE-C doses. The B16 cells were transfected with DNA/DMRIE-C either at a ratio of 1μg/2μl or 1μg/6μl. Supernatants were harvested 48h post-transfection. IL-2 production was measured by ELISA. Each bar represents the IL-2 production at 48h post-transfection. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments. *$p < 0.05$ when compared to the IL-2 level obtained from the pCMV-IL-2 control vector using DNA/DMRIE-C at 1ug/6ul. pHi1: pHi-1-IL-2 vector; pHi2: pHi-2-IL-2; pCMV: pCMV-IL-2; DC2: 2μl DMRIE-C and DC6: 6μl DMRIE-C

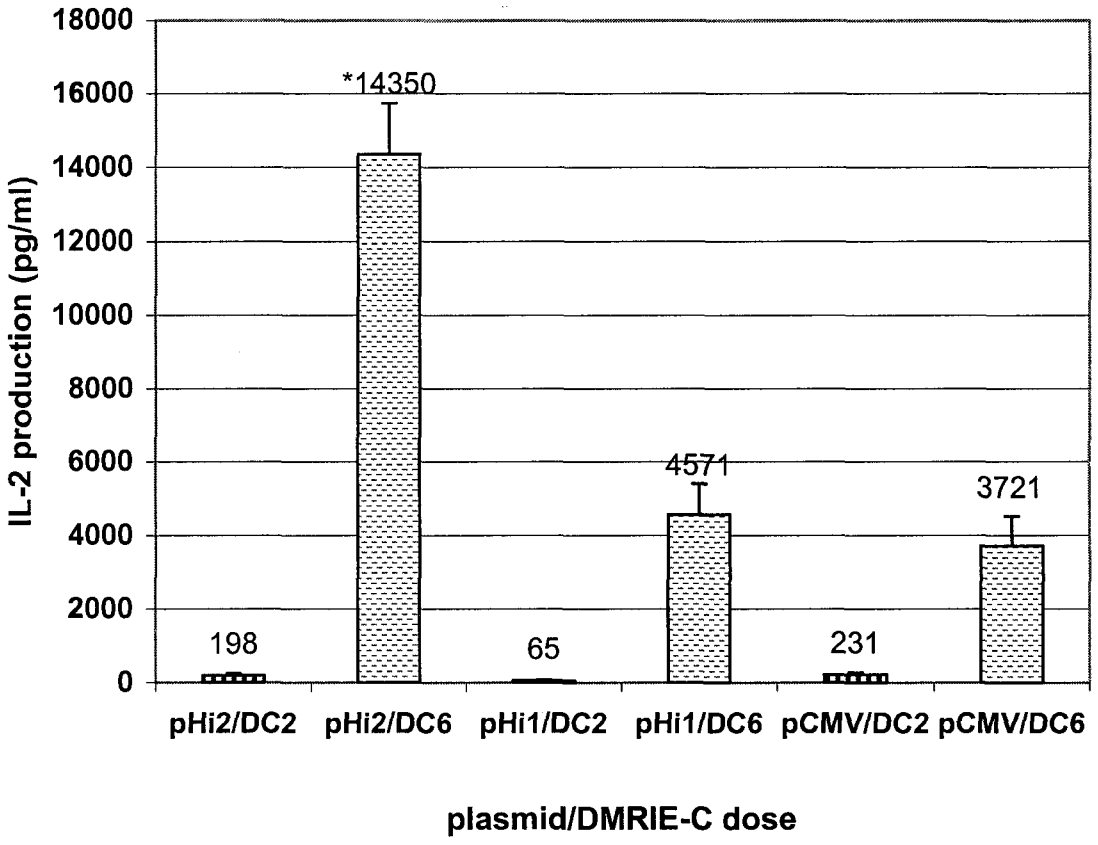


Figure 15. IL-2 production in LL/2 tumor cells using different DNA/DMRIE-C doses. The LL/2 cells were transfected with DNA/DMRIE-C either at a ratio of 1μg/2μl or 1μg/6μl. Supernatants were harvested 48h post-transfection. IL-2 production was measured by ELISA. Each bar represents the IL-2 production at 48h post-transfection. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments. *$p < 0.05$ when compared to the IL-2 level obtained from the pCMV-IL-2 control vector using DNA/DMRIE-C at 1μg/6μl. pHi1: pHi-1-IL-2 vector; pHi2: pHi-2-IL-2; pCMV: pCMV-IL-2; DC2: 2μl DMRIE-C and DC6: 6μl DMRIE-C

plasmid DNA/DMRIE-C (ratio)	cell line	cell number ± SEM	cell viability (%)
control	B16	$6.8 \pm 0.45 \times 10^5$	100
pHi-1-IL-2 (1ug/2ul) (1ug/6ul)	B16	$6.5 \pm 0.5 \times 10^5$ $6.4 \pm 0.45 \times 10^5$	96 94
pHi-2-IL-2 (1ug/2ul) (1ug/6ul)	B16	$6.8 \pm 0.65 \times 10^5$ $6.5 \pm 0.75 \times 10^5$	100 96
pCMV-IL-2 (1ug/2ul) (1ug/6ul)	B16	$6.75 \pm 0.5 \times 10^5$ $6.6 \pm 0.75 \times 10^5$	99 97
pHi-1-EGFP (1ug/2ul) (1ug/6ul)	B16	$6.4 \pm 0.5 \times 10^5$ $6.35 \pm 0.45 \times 10^5$	94 93
pHi-2-EGFP (1ug/2ul) (1ug/6ul)	B16	$6.65 \pm 0.45 \times 10^5$ $6.5 \pm 0.5 \times 10^5$	98 96
pCMV-EGFP (1ug/2ul) (1ug/6ul)	B16	$6.75 \pm 0.5 \times 10^5$ $6.5 \pm 0.65 \times 10^5$	99 96

Table 3. Cell viability in B16 cells transfected with DNA/DMRIE-C at different ratio. B16 cells were seeded at 2.0×10^5 cells/well in the 6-well plates. The pHi-1-IL-2, pHi-2-IL-2, pCMV-IL-2, pHi-1-EGFP, pHi-2-EGFP and pCMV-EGFP plasmids were transfected into the cells, respectively, 24h later. The non-transfected tumor cells were used as control. All cells were harvested 48h post-transfection and counted for cell viability. Each cell number represents the mean ± SEM of triplicate samples from one of the three independent experiments.

plasmid DNA/DMRIE-C (ratio)	cell line	cell number ± SEM	cell viability (%)
control	LL/2	$6.1 \pm 0.5 \times 10^5$	100
pHi-1-IL-2 (1ug/2ul) (1ug/6ul)	LL/2	$6.05 \pm 0.65 \times 10^5$ $6.0 \pm 0.5 \times 10^5$	99 98
pHi-2-IL-2 (1ug/2ul) (1ug/6ul)	LL/2	$5.95 \pm 0.75 \times 10^5$ $5.9 \pm 0.5 \times 10^5$	98 96
pCMV-IL-2 (1ug/2ul) (1ug/6ul)	LL/2	$6.05 \pm 0.5 \times 10^5$ $6.0 \pm 0.6 \times 10^5$	99 98
pHi-1-EGFP (1ug/2ul) (1ug/6ul)	LL/2	$6.05 \pm 0.5 \times 10^5$ $6.0 \pm 0.75 \times 10^5$	99 98
pHi-2-EGFP (1ug/2ul) (1ug/6ul)	LL/2	$5.95 \pm 0.65 \times 10^5$ $6.05 \pm 0.5 \times 10^5$	98 99
pCMV-EGFP (1ug/2ul) (1ug/6ul)	LL/2	$6.0 \pm 0.65 \times 10^5$ $5.95 \pm 0.45 \times 10^5$	98 98

Table 4. Cell viability in LL/2 cells transfected with DNA/DMRIE-C at different ratio. LL/2 cells were seeded at 2.0×10^5 cells/well in the 6-well plates. The pHi-1-IL-2, pHi-2-IL-2, pCMV-IL-2, pHi-1-EGFP, pHi-2-EGFP and pCMV-EGFP plasmids were transfected into the cells, respectively, 24h later. The non-transfected tumor cells were used as control. All cells were harvested 48h post-transfection and counted for cell viability. Each cell number represents the mean ± SEM of triplicate samples from one of the three independent experiments.

Determination of the transfection efficiency of the pHi-1/2 amplifier vectors in murine B16 and LL/2 tumor cells.

For both the B16 and LL/2 cell lines, the pHi-2-IL-2 vector produced more IL-2 as compared to the pHi-1-IL-2 vector, which only produced IL-2 comparable to the pCMV-IL-2 vector when a high DMRIE-C dose (6μl) was used. To determine whether this finding resulted from a decreased transfection efficiency, the pHi-1/2-EGFP amplifier vectors and the control pCMV-EGFP vector were transfected into the B16 and LL/2 tumor cell lines. Using the above-described protocol, each plasmid was transfected into these cell lines at a DNA/DMRIE-C ratio of 1μg/2μl and 1μg/6μl. At 48h post-transfection, the transfected samples were fixed in 500ul 1% paraformaldehyde (see Appendix C) for FACS analysis of EGFP expression. As shown in Figures 16 and 17, the DMRIE-C reagent did not result in high transfection levels in either the B16 or LL/2 cell line at either dose. The percentage of the EGFP-expressing B16 and LL/2 cells was much lower than those observed with the human A549 and MCF-7 cells. However, in both cell lines, using a DNA/DMRIE-C ratio of 1μg/6μl resulted in a relatively higher percentage of EGFP-expressing cells than when using DNA/DMRIE-C at a 1μg/2μl ratio (Figures 16 and 17). Under either transfection condition, no significant cell death was observed in either cell line (Table 3 and 4). In contrast to what was observed in human A549 and MCF-7 cell lines, the pCMV-EGFP control vector had a significantly higher transfection efficiency than either of the two pHi-1/2-EGFP plasmids in the murine B16 and LL/2 tumor cell lines. This

observation might explain why the pHi-1-IL-2 amplifier vectors produced lower or comparable amounts of IL-2 as those obtained from the control pCMV-IL-2 vector under these transfection conditions.

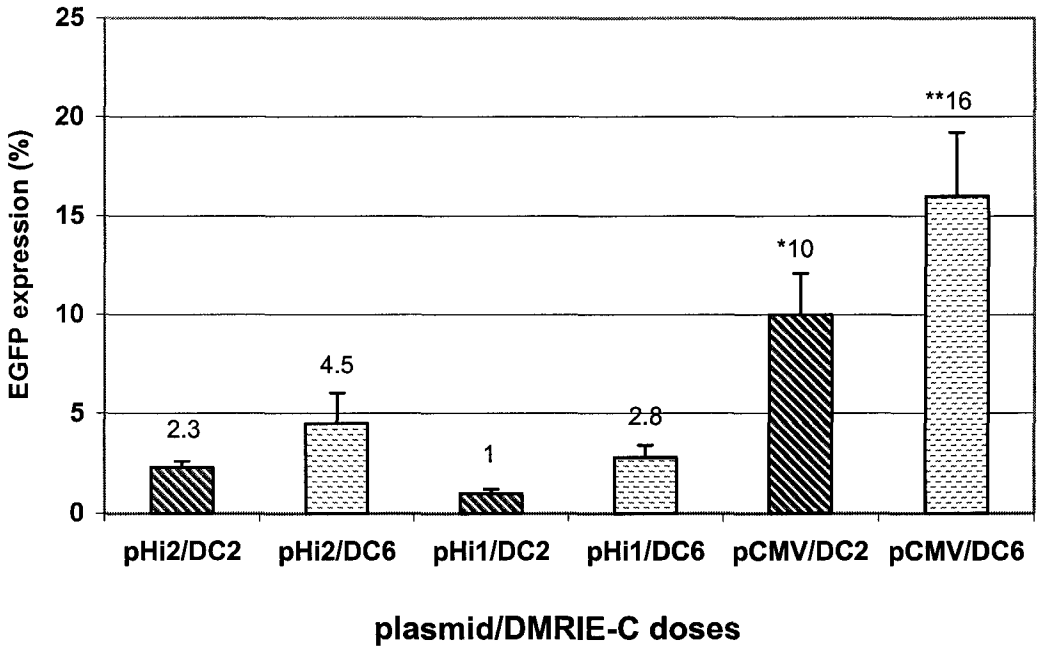


Figure 16. Percentage of EGFP-expressing B16 cells using different DNA/DMRIE-C ratios. The B16 cells were transfected with DNA/DMRIE-C either at a ratio of 1μg/2μl or 1μg/6μl. EGFP expressing cells were analyzed by FACS. Each bar represents the percentage of EGFP expressing cells. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments. *$p < 0.05$ when compared to the percentage of EGFP expressing cells obtained from the pHi-1-EGFP and pHi-2-EGFP vectors using DNA/DMRIE-C at 1μg/2μl. **$p < 0.05$ when compared to the percentage of EGFP expressing cells obtained from the pHi-1-EGFP and pHi-2-EGFP vectors using DNA/DMRIE-C at 1μg/6μl. pHi1: pHi-1-EGFP vector; pHi2: pHi-2-EGFP; pCMV: pCMV-EGFP; DC2: 2μl DMRIE-C and DC6: 6μl DMRIE-C

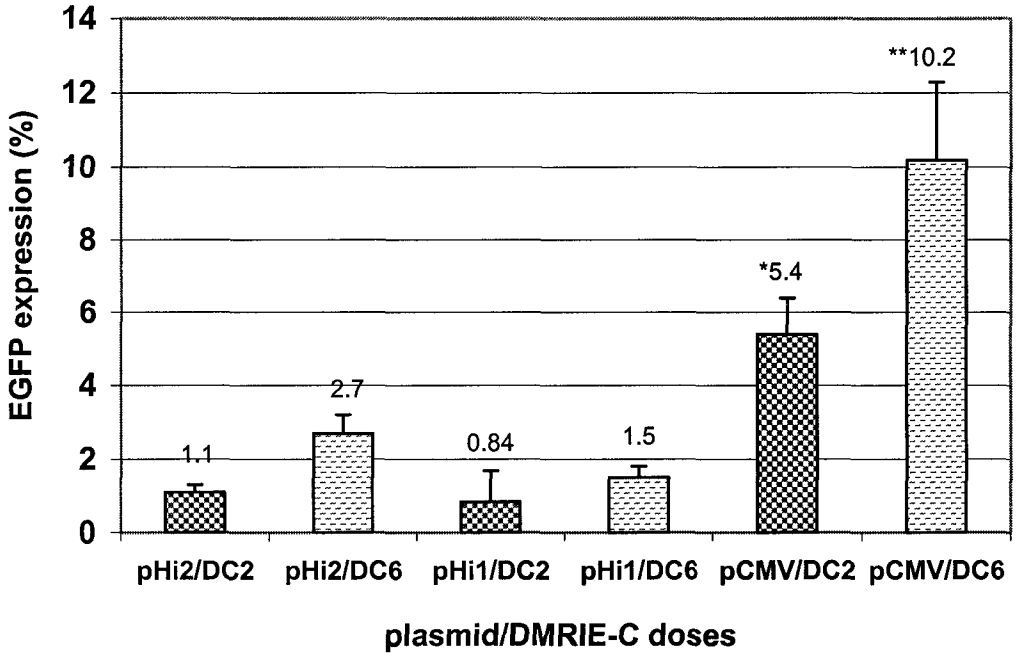


Figure 17. Percentage of EGFP-expressing LL/2 cells using different DNA/DMRIE-C ratios. The LL/2 cells were transfected with DNA/DMRIE-C either at a ratio of 1μg/2μul or 1μg/6μl. EGFP expressing cells were analyzed by FACS. Each bar represents the percentage of EGFP expressing cells. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments. *$p < 0.05$ when compared to the percentage of EGFP expressing cells obtained from the pHi-1-EGFP and pHi-2-EGFP vectors using DNA/DMRIE-C at 1μg/2μl. **$p < 0.05$ when compared to the percentage of EGFP expressing cells obtained from the pHi-1-EGFP and pHi-2-EGFP vectors using DNA/DMRIE-C at 1μg/6μl. pHi1: pHi-1-EGFP vector; pHi2: pHi-2-EGFP; pCMV: pCMV-EGFP; DC2: 2μl DMRIE-C and DC6: 6μl DMRIE-C

Generation of LL/2 clones stably transfected with pHi-2-IL-2 and control plasmids.

In all four cell lines tested, it was shown that the pHi-2-IL-2 vector produced more IL-2 than the pHi-1-IL-2 vector. The pHi-2-IL-2 vector was therefore chosen to test whether tumor cells producing higher levels of IL-2 would lead to decreased tumorigenicity in vivo. This study was first done by establishing pHi-2-IL-2 stably transfected tumor cell clones. To accomplish this, LL/2 cells were transfected with the pHi-2-IL-2 vector using DNA/DMRIE-C at a ratio of 1ug/6ul. The pCMV-IL-2, pHi-2-MCS and pCMV-MCS vectors were also transfected into LL/2 using the same transfection protocol. At 48h post-transfection, vector carrying LL/2 cells were selected in G418 (Gibco-BRL, Rockville, MD)-containing medium at a concentration of 1mg/ml. After two weeks of selection, 20 resistant colonies from each group of transfected cells were cloned by limiting dilution and expanded. All stably transfected clones were screened for IL-2 production. Briefly, each clone was seeded at 1.5 x 10^5 cells/well. 24h later, supernatant was collected for IL-2 production analysis by ELISA. In both the pHi-2-IL-2 and pCMV-IL-2 transfected groups, 5-6 clones from each group produced detectable levels of IL-2. The IL-2 production from clones transfected with the pHi-2-IL-2 vector ranged from 6000-90000 pg/ml whereas in the pCMV-IL-2 vector transfected group, it ranged from 300-1800 pg/ml (Figure 18). Three clones with the highest IL-2 production from each transfected group were chosen for study. Three pHi-2-MCS and three pCMV-MCS stably transfected clones were also chosen for analysis.

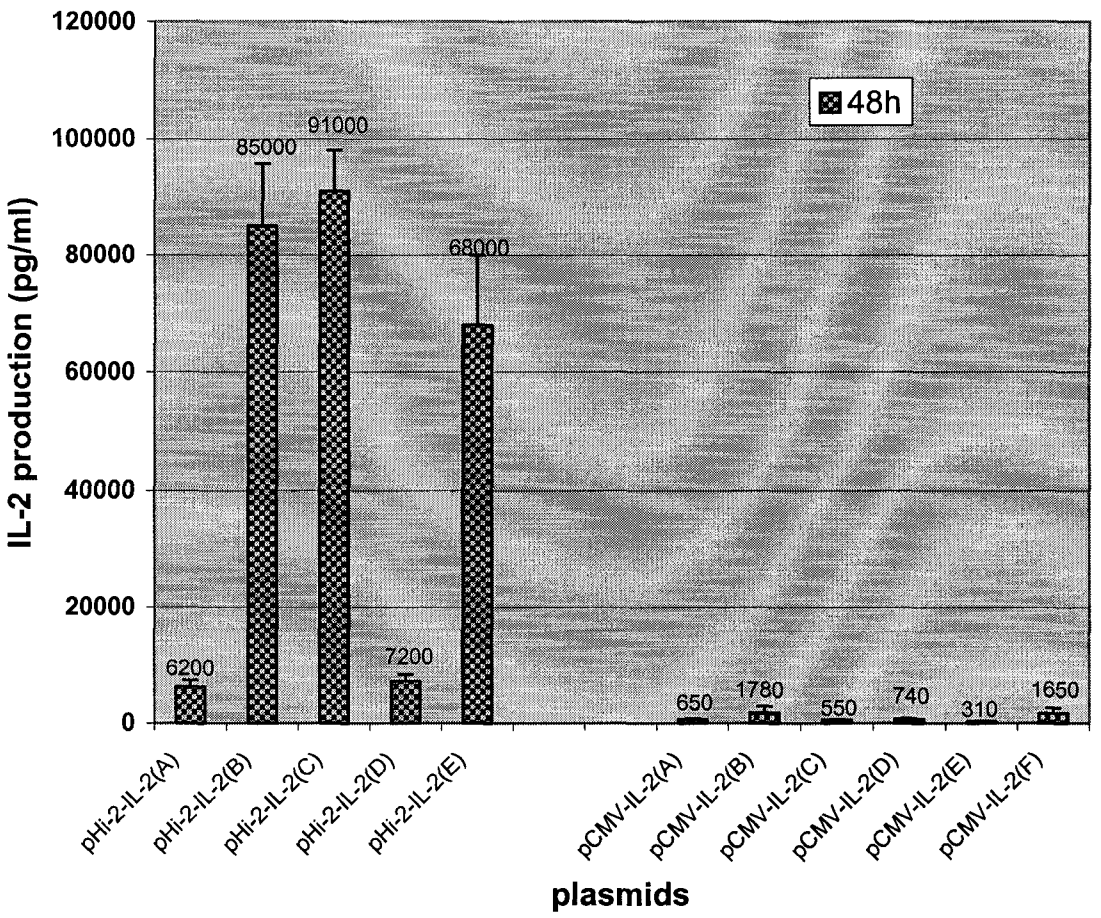


Figure 18. IL-2 production by LL/2 clones stably transfected with the pHi-2-IL-2 and pCMV-IL-2 vectors. The LL/2 cells were transfected with pHi-2-IL-2 and pCMV-IL-2 plasmids. Five (pHi-2-IL-2 A-E) of 20 stable clones from pHi-2-IL-2 transfected group and six (pCMV-IL-2 A-F) of 21 stable clones from the pCMV-IL-2 transfected group were obtained that produced detectable IL-2. Cells were seeded at 1 x 10^5 cells/well. Supernatants were harvested 48h after seeding. IL-2 production was measured by ELISA. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments.

In vitro growth characteristics of LL/2 tumor clones stably transfected with the different plasmids.

The in vitro growth rate of the chosen IL-2 expressing clones and vector backbone-transfected stable clones were characterized and compared to the parental LL/2 tumor cells. Briefly, each chosen clone was seeded at 1.5 x 10^5 cells/well in a 6-well plate in the absence of G418. Cell numbers from each well were counted and compared to their parental LL/2 tumor cells 48h later. Among the tested clones, the one from each stably transfected group that had a comparable growth rate to the parental LL/2 tumor cells was chosen for in vivo animal studies.

Table 5 demonstrates that the selected IL-2 expressing stable LL/2 clones had a similar growth rate as compared to the vector backbone-transfected clones and the parental LL/2 cells. A two day culture of 1x10^5 cells of the pHi-2-IL-2 and pCMV-IL-2 stably LL/2 clones resulted in a final yield of 5.2 ± 0.6 x 10^5 and 5.2 ± 0.75 x 10^5 cells, respectively. Similarily, 1x10^5 cells of pHi-2-MCS and pCMV-MCS vector backbone-transfected stable LL/2 clones and wild type LL/2 tumor cells proliferated to yield 5.1~ 5.4 ± 0.5 x 10^5 cells after 48h of culture. Table 5 shows the respective IL-2 production from each of the chosen clones 48h after being seeded.

Stable Clones	IL-2 production (pg/ml)	Cell number	SEM
pHi-2-IL-2 (B)	85000	5.2×10^5	0.6×10^5
pCMV-IL-2 (F)	1650	5.2×10^5	0.75×10^5
pHi-MCS	0	5.4×10^5	0.5×10^5
pCMV-MCS	0	5.1×10^5	0.5×10^5
wt LL/2	0	5.1×10^5	0.45×10^5

Table 5. In vitro comparison of IL-2 production and growth rate of LL/2 tumor clones stably transfected with the different plasmids. The pHi-2-IL-2, pHi-2-MCS, pCMV-IL-2 and pCMV-MCS stably transfected LL/2 tumor clones were seeded at 1×10^5 cells/2ml. The wild type (wt) LL/2 tumor cells were seeded at the same number as a control. Cells were harvested and counted 48h later. Each cell number represents the mean ± SEM of triplicate samples. Each clone present in the table was the clone chosen from the 3 tested clones from each transfected group which showed a similar growth rate to the wild type LL/2 cells. The corresponding IL-2 production is shown. pHi-2-IL-2 (B): clone B from 5 (A-E) of the pHi-2-IL-2 stably transfected LL/2 clones; pCMV-IL-2 (F): clone F from 6 (A-F) of the pCMV-IL-2 stably transfected LL/2 clones. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments.

Determination of the tumorigenicity of LL/2 tumor clones stably transfected with the different plasmids.

Having demonstrated the IL-2 expression levels and the growth rates of the gene-modified LL/2 tumor clones, we then determined the effect of the IL-2 expression levels on the tumorigenicity of each clone in vivo using the Lewis Lung carcinoma model. Briefly, C57BL/6 mice were injected with parental LL/2 tumor cells and each of the above-mentioned plasmid-modified LL/2 clones. Tumor development was monitored over a 27 day period and/or until the first mouse died. Tumors became palpable 7 days after injection with wild type LL/2 tumor cells and reached an average tumor volume of 1492 ± 115 mm^3 (Figure 19) by day 25. Although the pHi-2-IL-2 modified LL/2 tumors were also palpable 7 days after tumor cell injection and reached an average tumor volume of 335 ± 115 mm^3, the growth of these clones was significantly slower as compared to the growth of pCMV-IL-2-modified LL/2 clones, vector backbone-transfected clones and wild type LL/2 tumor cells. Tumor growth in mice injected with pCMV-IL-2 modified clones was only slightly slower than tumor growth in mice injected with vector backbone-transfected clones or parental LL/2 tumor cells. The pCMV-IL-2 modified tumor group reached an average tumor volume of 1210 ± 369 mm^3 (Figure 19) by day 25.

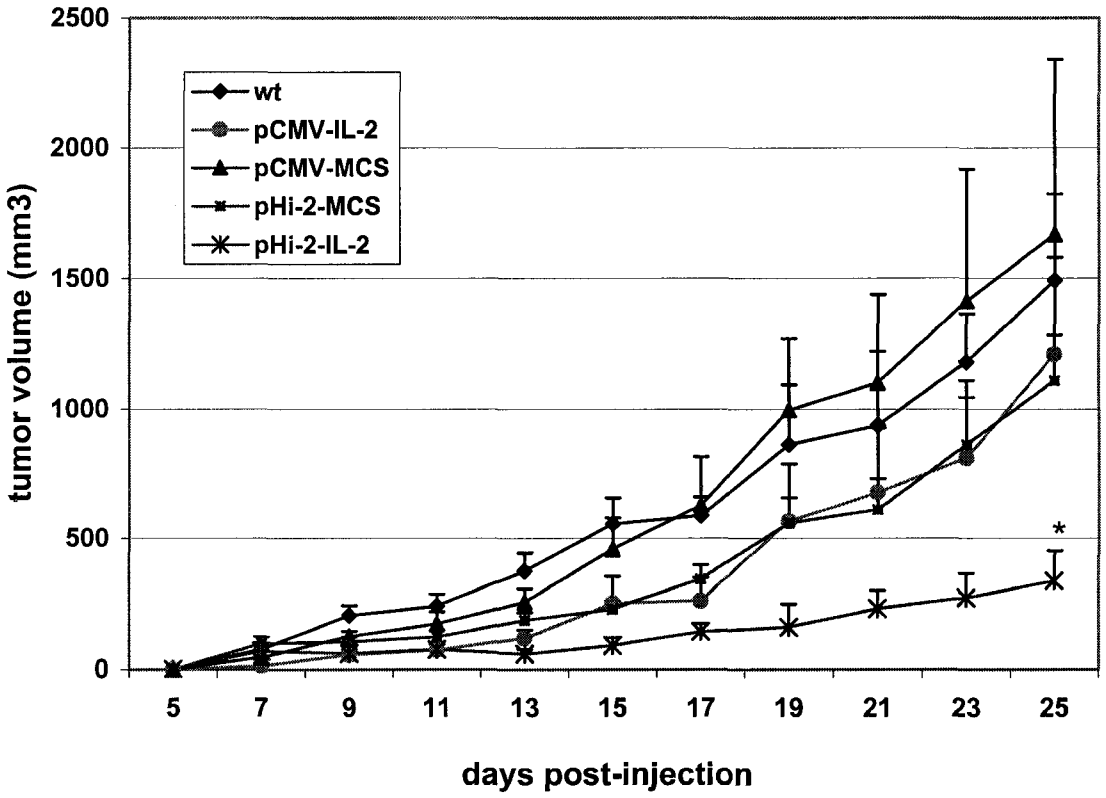


Figure 19. Tumor growth in C57BL/6 mice after injection with the different plasmids stably transfected LL/2 clones. C57BL/6 mice were injected with pHi-2-IL-2 and pCMV-IL-2 stably transfected Lewis Lung (LL/2) clones. Mice were also injected with pHi-2-MCS and pCMV-MCS stably transfected clones, and the parental LL/2 tumor cells as controls. Each group contained 8 mice and each mouse was injected with 0.5 x 10^6 tumor cells subcutaneously. The tumorigenicity of the different tumor cells was monitored by measuring primary tumor growth. Tumor growth was monitored every other day from day 5 post-injection for 25 days. Each line represents the average tumor volume ± SEM of 8 mice in each individual group. (*denotes significantly different tumor volumes compared to the control group injected with pCMV-IL-2 vector-modified tumor clone; $p < 0.05$). Data shown are the results from one of three experiments.

Determination of the survival rate of mice injected with LL/2 tumor clones stably transfected with different plasmids

To determine whether the high IL-2 producing clones decreased the in vivo tumorigenicity and led to increased survival, the survival rate of mice injected with the different LL/2 tumor clones was evaluated and compared to animals injected with the parental tumor cells. Figure 20 shows that the group of mice injected with the pHi-2-IL-2-modified clones had significantly longer survival as compared to the groups injected with the other clones. The group of mice injected with the pCMV-IL-2 modified clones did not exhibit significantly increased survival as compared to mice injected with either vector backbone-transfected clones or parental tumor cells. In this study, the longer survival correlated with the observed slower tumor growth rate in these mice. This finding indicated that higher IL-2 production from the pHi-2-IL-2 amplifier vector-modified LL/2 tumor clones decreased the tumorigenicity in vivo and thus led to longer survival.

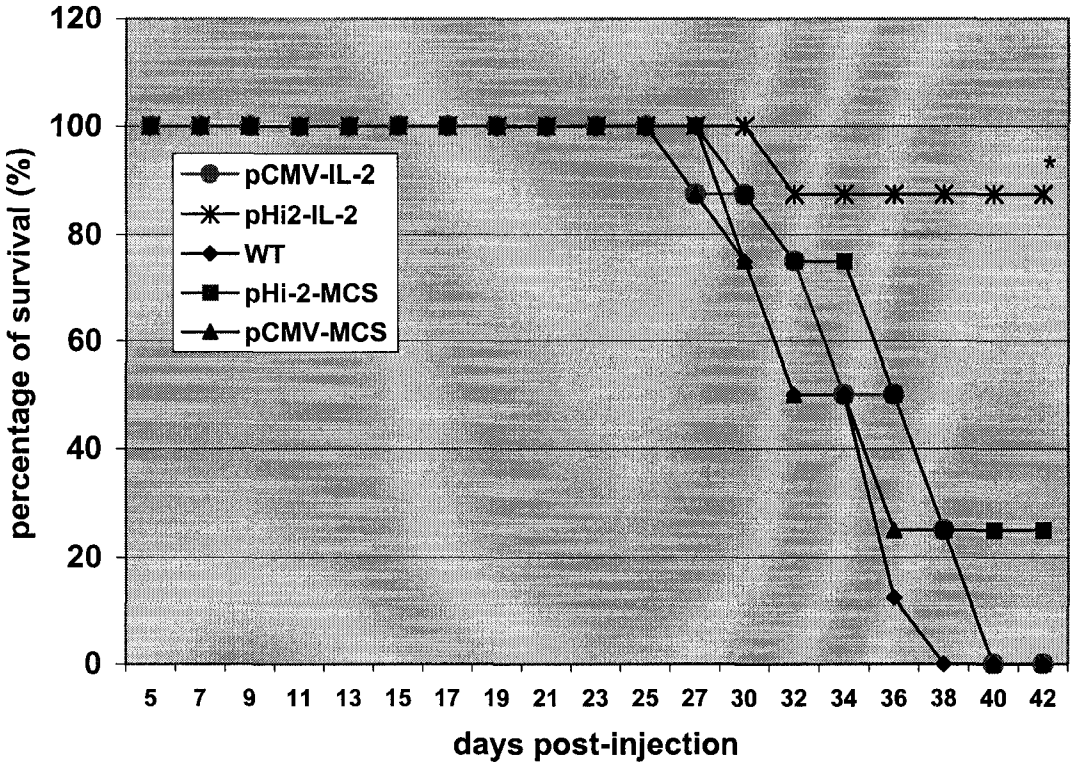


Figure 20. Survival rate of C57BL/6 mice after injection with different plasmids stably transfected LL/2 clones. Survival rate in mice injected with pHi-2-IL-2, pCMV-IL-2, pHi-2-MCS, pCMV-MCS stably transfected Lewis Lung (LL/2) clones and parental LL/2 cells was monitored and compared between these groups. Each group contained 8 mice and each mouse was injected with 0.5 x 10^6 tumor cells subcutaneously. All mice were sacrificed because of excessive tumor burden in the group of mice injected with the pHi-2-MCS modified tumor clones. Individual lines represent the percent survival for each group. *$p < 0.05$ when compared to other groups. Data shown are the results from one of three experiments.

Determination of serum IL-2 levels in mice injected with LL/2 tumor clones stably transfected with different plasmids.

To determine whether injection of the pHi-2-IL-2 plasmid-modified LL/2 stable tumor cell clones could lead to higher IL-2 levels in vivo, the IL-2 levels in sera of mice from each group after tumor cell injection was measured. Mice were injected with pHi-2-IL-2, pCMV-IL-2, pHi-2-MCS, pCMV-MCS vector-modified, stable clones and the parental tumor cells. Serum IL-2 levels were monitored at days 1, 3, 7, and 10 after tumor cell injection. The difference in efficacy between these vectors was found to be correlated to the amount of IL-2 produced, which reached 55.3 ± 15.8 pg/ml for the pHi-2-IL-2 vector and 5.8 ± 2.5 pg/ml for the pCMV-IL-2 vector at day 1 post-injection (Figure 21). Serum IL-2 expression declined very rapidly in both groups. Serum IL-2 was still detectable (7.0 ± 3.6 pg/ml) at day 3 in mice injected with the pHi-2-IL-2 stably transfected tumor cell clones whereas IL-2 was not detectable in mice injected with the pCMV-IL-2 vector stably transfected tumor cell clones at this time point. Serum IL-2 was not detectable in mice injected with pHi-2-MCS or pCMV-MCS vector-modified tumor clones, or with parental tumor cells during the 10-day period.

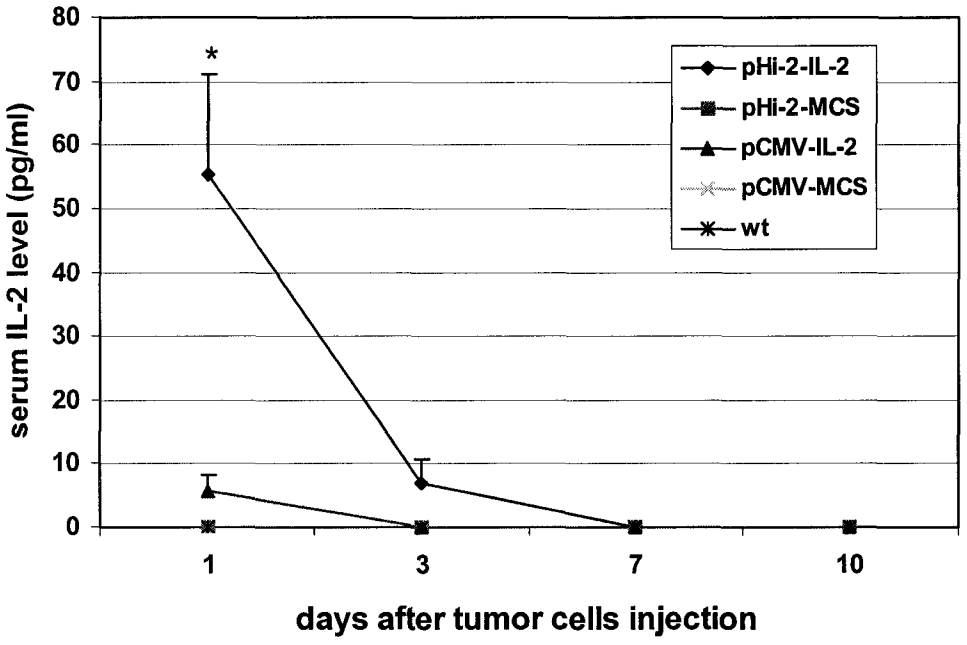


Figure 21. Serum IL-2 concentrations in mice after injection with different vector-modified LL/2 stable tumor cell clones. C57BL/6 mice were injected with pHi-2-IL-2 and pCMV-IL-2 stably transfected Lewis Lung (LL/2) clones. Mice were also injected with pHi-2-MCS and pCMV-MCS stably transfected clones, and the parental LL/2 tumor cells as controls. Each group contained 4 mice and each mouse was injected with 0.5 x 10^6 tumor cells subcutaneously. Serum IL-2 levels were monitored at day 1,3,7 and 10 after tumor cells injection. Data are as average of 4 mice in each group ± SEM from one experiment. *$p < 0.05$ as compared with control groups.

CHAPTER 4

DEVELOPMENT OF A NOVEL INDUCIBLE AND HIGH GENE EXPRESSION PLASMID FOR SAFE AND EFFECTIVE CANCER GENE THERAPY

4.1 MATERIALS AND METHODS

Mice

Six- to eight-week old female C57BL/6, H-2^b mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed at the University of Arizona Animal Facilities in accordance with the principles of animal care (NIH publication No. 85-23, revised 1985)

Cell lines

Human lung cancer (A549) cells, human breast cancer (MCF-7) cells, mouse melanoma (B16) cells and mouse Lewis Lung carcinoma (LL/2) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproducts, Calabasas, CA), penicillin-streptomycin and gentamycin (Gibco-BRL, Rockville, MD). Cells were grown in an atmosphere of 5% CO_2 at 37°C and subcultured every 2-3 days using 0.05% trypsin.

Construction of the pHot-IL-2-nk plasmid

The pHot-IL-2-nk plasmid was constructed by inserting the EcoR I digested human IL-2 gene fragment from pSac-KiSS-IL-2 (40) into the EcoR I site of pHot-MCS-nk. The pHot-MCS-nk plasmid was generated by ligating the 3.2 kb Xho I-Hpa I fragment of pEGFP-1 (Clontech, Palo Alto) with a 0.7 kb Sal I-Pvu II fragment from pHot-1-MCS. The pHot-1-MCS plasmid was generated by replacing the human cytomegalovirus (CMV) promoter of pcDNA3.1 (Invitrogen, San Diego, CA) with a 451 bp BamH I-Hind III fragment of the human HSP70B promoter from the p173OR plasmid (StressGen, Victoria, BC, Canada).

Construction of the pHi-Hot-IL-2-nk plasmid

The pHi-Hot-IL-2-nk plasmid was constructed by inserting the EcoR I digested human IL-2 gene fragment from pSac-KiSS-IL-2 (40) into the EcoR I site of pHi-Hot-MCS-nk. The pHi-Hot-MCS-nk plasmid was created by ligating a 1.0 kb Bgl II-EcoR I fragment from pHIV2-neo (40) with pHot-TAT-nk. The pHot-TAT-nk plasmid was created by inserting a 0.9 kb Kpn I-BamH I fragment from pHi-1-MCS (data unpublished) into pHot-MCS-nk.

Construction of the pCMV-IL-2-nk plasmid

The pCMV-IL-2-nk plasmid was generated by inserting the EcoR I digested human IL-2 gene fragment from pSac-KiSS-IL-2 (40) into the EcoR I site of pCMV-MCS-nk. The pCMV-MCS-nk plasmid was generated by ligating a 3.4 kb

Bgl II-Hind III fragment from pHot-MCS-nk with a 0.9 kb Bgl II-Hind III fragment from pcDNA3.1.

In vitro transfections using the DMRIE-C reagent

Cells to be transfected were seeded at $2x10^5$ cells/well in a six well tissue culture plate in 2ml complete RPMI 1640 medium and incubated at 37°C until the cells were 40–60% confluent. The DMRIE-C reagent (Invitrogen, Carlsbad, CA) was used for all transfections. In Chapter 3, using different DNA/DMRIE-C ratios, the optimal transfection conditions for each of the four cell lines were characterized and described. We also observed that the human A549 and MCF-7 cells lines were more sensitive to the toxicity of high dose DMRIE-C mediated transfection than the murine B16 and LL/2 cell lines. Thus, in this study, DNA/lipid ratios of 1μg/2μl for the human cells and 1μg/6μl for the mouse cells were used for all transfections. For each well, 1μg of plasmid DNA was suspended in 500μl OPTI Reduced Serum Medium (Gibco-BRL, Rockville, MD). Either 2μl or 6μl DMRIE-C lipid was suspended in 500μl OPTI medium. The DNA and DMRIE-C solutions were mixed together and incubated at room temperature for 30 minutes. Cells were then washed with OPTI medium and incubated with 1ml of the DNA/lipid mixture at 37°C. After 4h, the media was replaced with 2ml RPMI culture medium. Supernatants from the cultures were harvested every 24h post-transfection and stored at –80°C until analyzed. Medium was then renewed completely with fresh medium. All transfections were done in triplicate.

Establishment of LL/2 tumor clones stably transfected with the different plasmids.

The pHi-Hot-IL-2-nk, pHot-IL-2-nk and pCMV-IL-2-nk vectors were transfected into LL/2 cells. Forty-eight hours after transfection, the transfected cells were seeded into 96-well tissue culture plates (Falcon, Becton Dickinson labware: Lincoln Park, NJ) at 500-800cells/well. Vector-carrying clones were selected in 200μl G418 (Gibco-BRL, Rockville, MD) containing medium at a concentration of 1mg/ml. After 2 weeks approximately 20 drug-resistant colonies from each transfected group were cloned by limiting dilution and expanded for future experiments. All isolated clones were screened for basal IL-2 expression and then frozen at –80°C for future study. The clones with the highest IL-2 expression from each transfected group was chosen for the repeated heat-induction experiments.

***In vitro* heat shock treatment**

Cells were heated at 24h post-transfection after culture supernatants had been collected and replaced with fresh culture medium. The 6-well plates were sealed with parafilm and then immersed in a Precision Dual Chamber waterbath (Lehman Scientific Wrightsville, PA) at the pre-set heating temperature of 42°C for 30 min. After the heat shock treatment, the 6-well plates were immediately returned to the 37°C incubator under the culture conditions described above. Similarily, LL/2 stably transfected clones from the heat shock groups were heated at 42°C for 30 min after seeding for 24h and 72h, respectively. Supernatants

were collected and replaced with fresh culture medium every 24h. The heat shock treatments were performed immediately after the supernatants were collected.

IL-2 ELISA

IL-2 secretion into the supernatants was measured with a human IL-2 ELISA kit (Pharmingen, San Diego, CA) using protocols supplied by the manufacturer. Briefly, a 96-well microtitre plate was coated with capture monoclonal antibodies specific for human IL-2. 100ul of supernatant from each of triplicate samples was added to the wells, either as is or diluted, and incubated for 2 hours at room temperature. The wells were then rinsed several times with washing buffer prior to addition of the detection antibody conjugated to horseradish peroxidase. The plate was incubated for an additional 1h at room temperature, the wells were washed as before, and 100ul of substrate solution was added to each well. After 30 min at room temperature, the reaction was stopped by the addition of 2N H_2SO_4. The plate was read on a microplate reader at an absorbance of 450nm. A standard curve was plotted using recombinant IL-2 provided in the kit and the IL-2 concentrations were determined by extrapolation from the standard curve. Results were calculated as pg/ml of IL-2.

In vivo heat shock treatment

In vivo heat shock treatment was started at day 3 after tumor cell injection. Each mouse in the heat group was put into a 50ml tissue culture tube (VWR Scientific products, Willard, OH) with holes punched into it which allowed heated water to enter. The culture tube with the mouse was then put into the Precision Dual Chamber waterbath (Lehman Scientific Wrightsville, PA) at a pre-set heating temperature of either 42°C or 43°C for 30min. The tubes were put into the waterbath at a level that immersed half of the mouse's body in the water during the heat treatment. After heat shock treatment, the mice were dried with a hair dryer and placed back into their cages.

Determination of the effect of heat shock on the tumorigenicity of pHi-Hot-IL-2-nk stably transfected LL/2 clones

The tumorigenicity of pHi-Hot-IL-2-nk plasmid stably transfected LL/2 clones and parental LL/2 tumor cells was determined by monitoring primary tumor growth in syngeneic mice. Primary tumors were generated by subcutaneous injection of 0.5×10^6 viable tumor cells in 100ul PBS in the hind flank of each C57BL/6 mouse. Tumors were measured in two perpendicular dimensions every other day for 17 days using vernier calipers (VWR Scientific products, Willard, OH). Tumor volume was calculated using the formula $v=(l)(w^2)/2$, where v = volume (mm^3), l = long diameter, and w = short diameter.

Statistical Analysis

Results are given as the mean ± stand errors of the mean (SEM). Differences between groups in vitro tests and differences in tumor diameter in experiments in vivo were analyzed for significance by student's t-test (two-tailed). Statistical significance was set at $p < 0.05$.

4.2 RESULTS

Design and construction of a novel inducible, amplifier pHi-Hot plasmid for high and controlled transgene expression.

For safe and effective gene therapy, the development of new expression vectors with high and controlled expression properties is an attractive solution and has broad applications. To this end, a novel inducible and high expression system, pHi-Hot-MCS-nk, was designed and constructed by combining the inducible and amplifier strategies into a single construct.

The pHi-Hot-MCS-nk vector (Figure 22) makes use of three independent transcriptional units, rather than using a viral internal ribosomal entry site (IRES). IRES usually result in much lower co-expression of the gene put behind it (87,88). The first transcriptional unit contains a gene encoding the transcriptional activator, Tat, driven by an inducible hsp70B promoter. The second transcriptional unit contains an HIV2 LTR promoter driving the gene of interest. The HIV2 LTR promoter activity would therefore be enhanced by the amplifier (Tat) present in the same construct which should lead to increased expression level of the second gene. Using the hsp promoter to drive the Tat gene, the magnitude and duration of the amplified second gene expression can be regulated by controlling the hsp promoter activity. The third transcriptional unit contains the neomycin/kanamycin resistance gene under the control of the

bacterial Ampicillin gene promoter and the SV40 promoter to allow for selection in both mammalian cells and bacteria. Transgenes of interest can be directly cloned into the pHi-Hot-MCS-nk vector at the compatible restriction sites present in its multiple cloning site (MCS) downstream of the HIV2 LTR promoter. This large MCS contains 8 commonly used restriction sites that allow convenient insertion of the gene(s) into pHi-Hot-MCS-nk. In this study, the human IL-2 cytokine gene was used as a reporter gene. Two control constructs were made using the same backbone in which the IL-2 gene was under the direct control of either the hsp promoter or the CMV promoter (Figures 23 and 24).

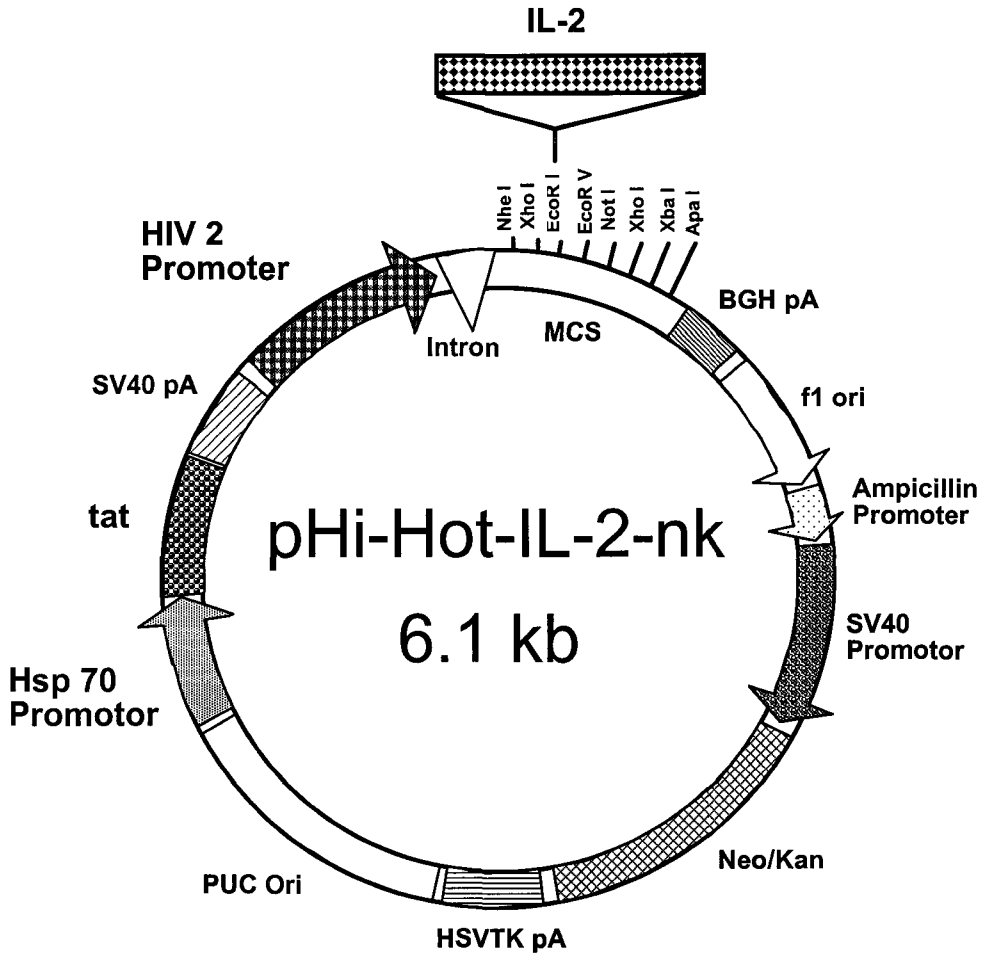


Figure 22. Diagrammatic representation of the pHi-Hot-IL-2-nk plasmid. The first gene encodes a transcriptional activator (Tat) driven by an inducible hsp70B promoter. The second promoter (HIV2 LTR) drives the human IL-2 cytokine gene. nk: neomycin/kanamycin resistant gene

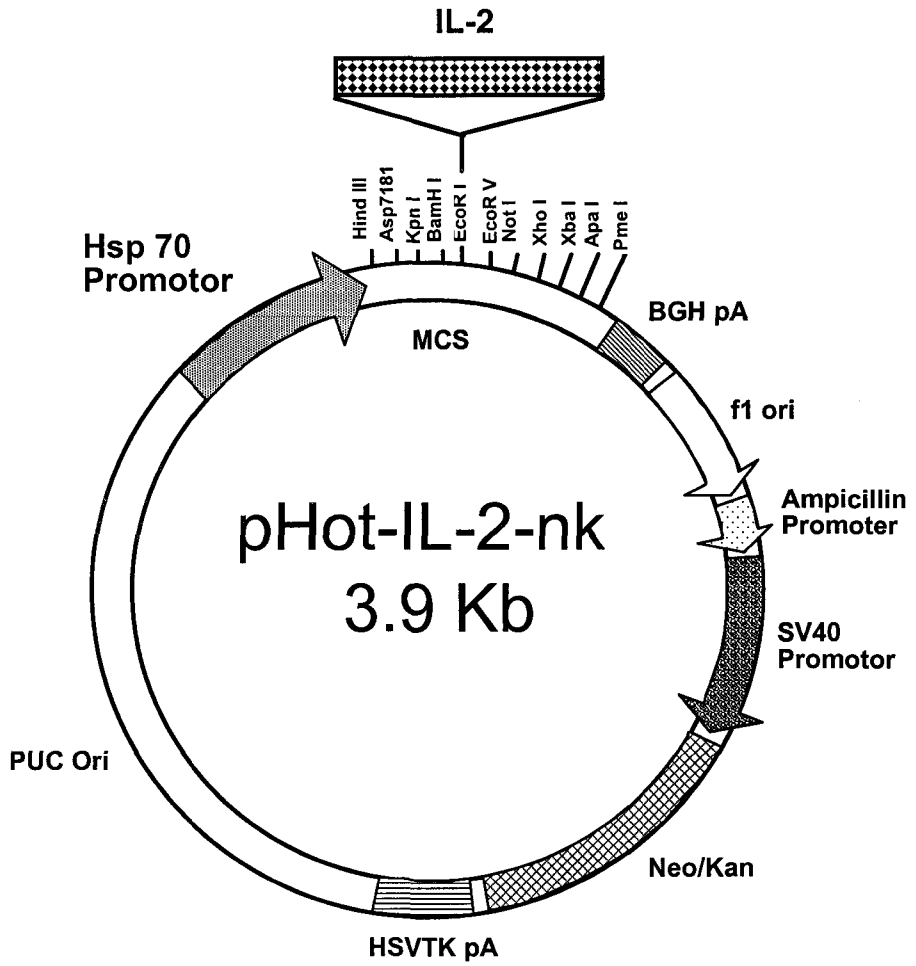


Figure 23. Diagrammatic representation of the pHot-IL-2-nk plasmid. The inducible heat shock promoter directly drives the human cytokine IL-2 gene. nk: neomycin/kanamycin resistant gene

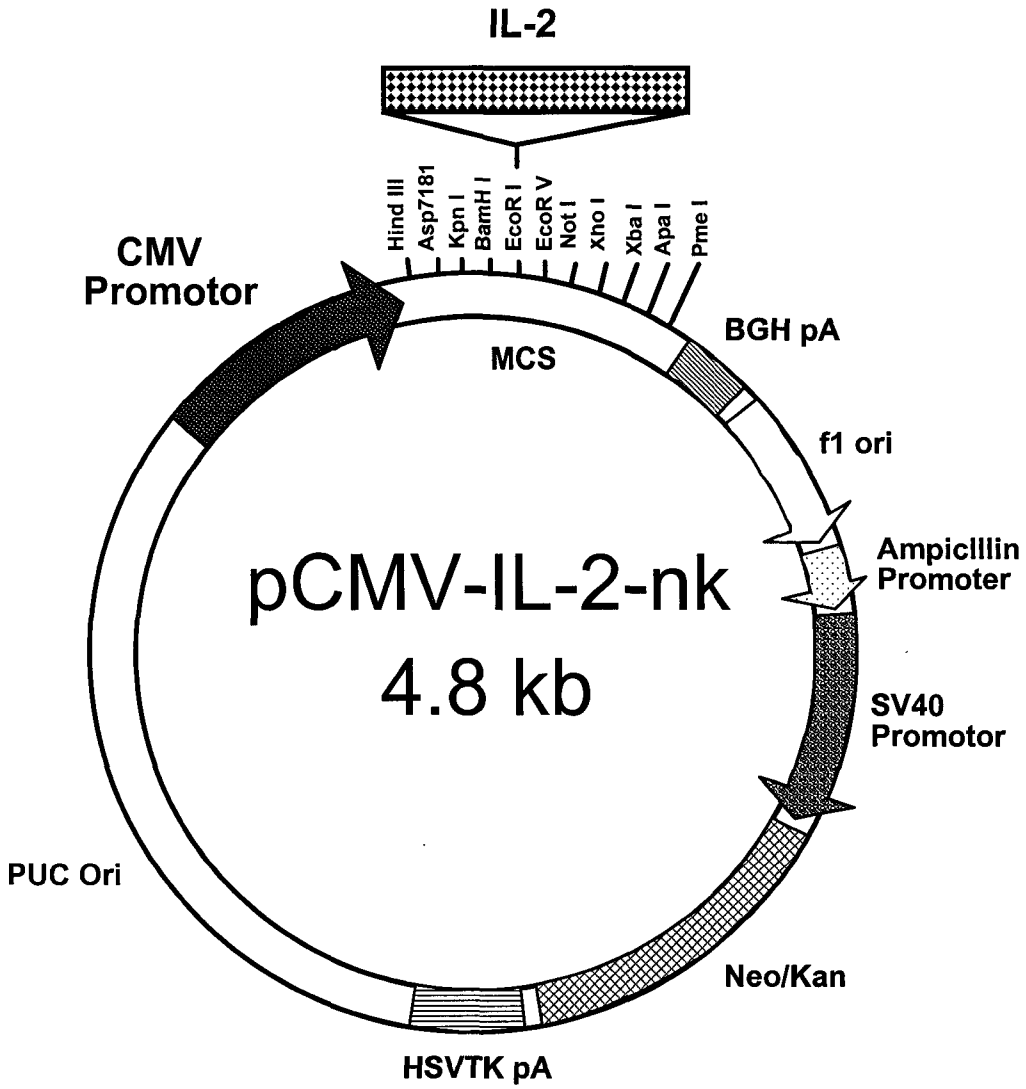


Figure 24. Diagrammatic representation of the pCMV-IL-2-nk plasmid. The constitutive CMV promoter directly drives the human cytokine IL-2 gene. nk: neomycin/kanamycin resistant gene

Determination of heat shock promoter activity under different heating conditions.

The DMRIE-C reagent was used for all transfections in this study. To evaluate the impact of different heating conditions on hsp promoter activity, human breast cancer (MCF-7) cells were transfected with the pHot-IL-2-nk plasmid, in which the IL-2 gene was under direct control of the hsp promoter. At 24h post-transfection, the MCF-7 cells were heated for either 30 min or 60 min at temperatures ranging from 41- 43°C in 1°C increments. As shown in Figure 25, for each condition tested, 24h after heat shock (48h post-transfection) IL-2 production from pHot-IL-2-nk was significantly increased as compared to its unheated control. It then dropped back to baseline levels within the next 24h. Increases in IL-2 induction were correlated with temperature elevation and length of heat shock. This finding was similar to observations from other investigators that hsp promoter activity increased with the magnitude and duration of heating temperatures (59,92). Borreli *et al* have reported that maximal transgene expression could be achieved using a heat shock at 42-43°C for 20-30 min. Further, heat induction at temperatures higher than 43.0°C produced progressively lower transgene expression because the harsher heat shocks caused significant cell death (59). Under our experimental conditions, heat shock at 43°C did not further increase IL-2 production as compared to heat shock at 42°C. Longer heat shocks at either temperature did not significantly yield more IL-2 production. Since heating at 42°C for 30 min was able to induce nearly

maximal IL-2 production driven by the hsp promoter with minimal cell death in MCF-7 cells (Figure 25), this heating condition was used as the standard heat shock treatment in all subsequent experiments.

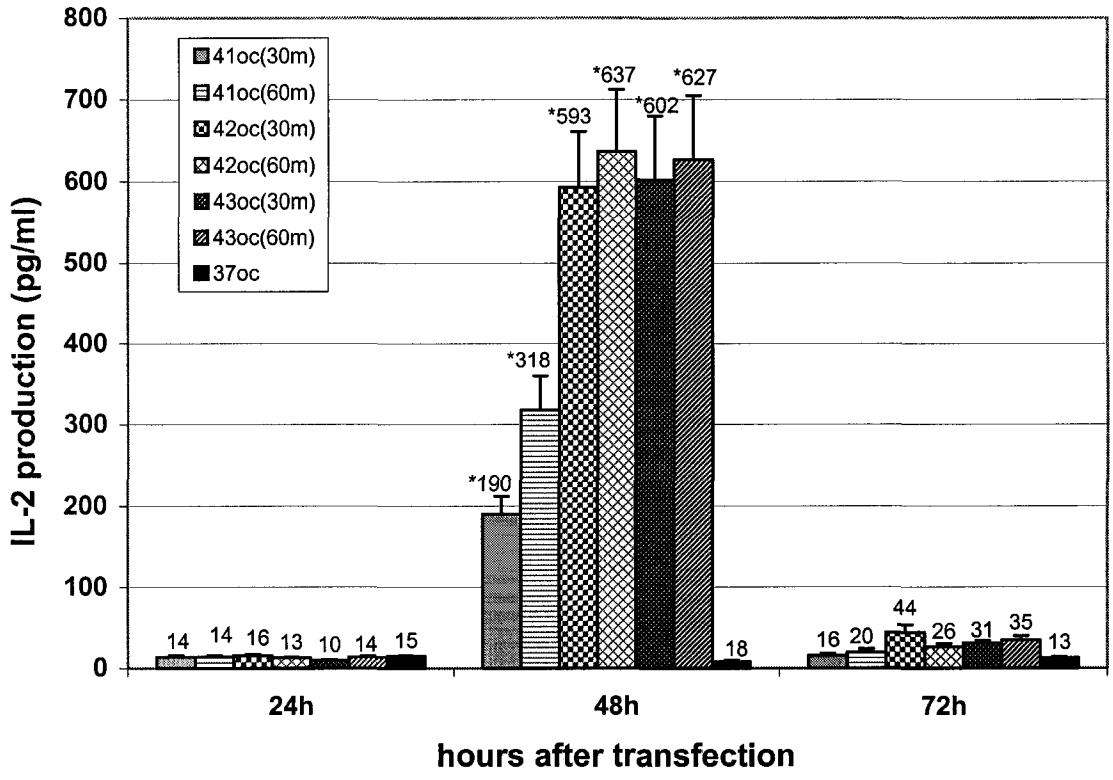


Figure 25. IL-2 production by the pHot-IL-2-nk plasmid in MCF-7 cells under diffrent heat shock conditions. 24h after transfection cells were heated at three different temperatures (41°C, 42°C and 43°C) for 30 min or 60 min, respectively. Supernatants were harvested every 24h for 3 consecutive days. IL-2 production was measured by ELISA. Each bar represents the IL-2 production within every 24h period after transfection. The values shown are the means of triplicate determinations ± SEM from one of three independent experiments. *$p < 0.005$ when compared to its unheated control (37°C).

Determination of heat induction of IL-2 secretion from the pHi-Hot-IL-2-nk plasmid in human A549 and MCF-7 tumor cells.

To determine the basal and heat-induced levels of IL-2 expression from the pHi-Hot-IL-2-nk plasmid, this vector was transfected into A549 and MCF-7 cells. The pHot-IL-2-nk and pCMV-IL-2-nk vectors were used as controls to compare the IL-2 expression levels to those achieved from using either the hsp or the CMV promoters directly. Figure 26 shows that, in A549 cells, 24h after moderate heat treatment at 42°C for 30 min, transient IL-2 production by the pHi-Hot-IL-2-nk plasmid was induced to 4056 ± 469 pg/ml, which was a 3-fold increase as compared to its unheated control (1305 ± 143 pg/ml). This induced level of IL-2 was 72 and 12-fold higher than that observed with the pHot-IL-2-nk (56 ± 8 pg/ml) and the pCMV-IL-2-nk (341 ± 38 pg/ml) control vectors, respectively (Figure 26). A similar pattern of IL-2 production was observed in MCF-7 cells transfected with the pHi-Hot-IL-2-nk plasmid. After heat treatment, transient IL-2 production increased from 1100 ± 168 pg/ml to 6082 ± 1279 pg/ml over the next 24h, which was 38-fold higher than that of the pHot-IL-2-nk vector (405 ± 39 pg/ml), and 10-fold higher than that of the pCMV-IL-2-nk vector (591± 65 pg/ml) (Figure 27). In both cell lines IL-2 secrection from the pHi-Hot-IL-2-nk vector dropped back to basal levels in the following 24h. IL-2 expression driven by the CMV promoter was only slightly elevated after heat treatment (Figures 26 and 27).

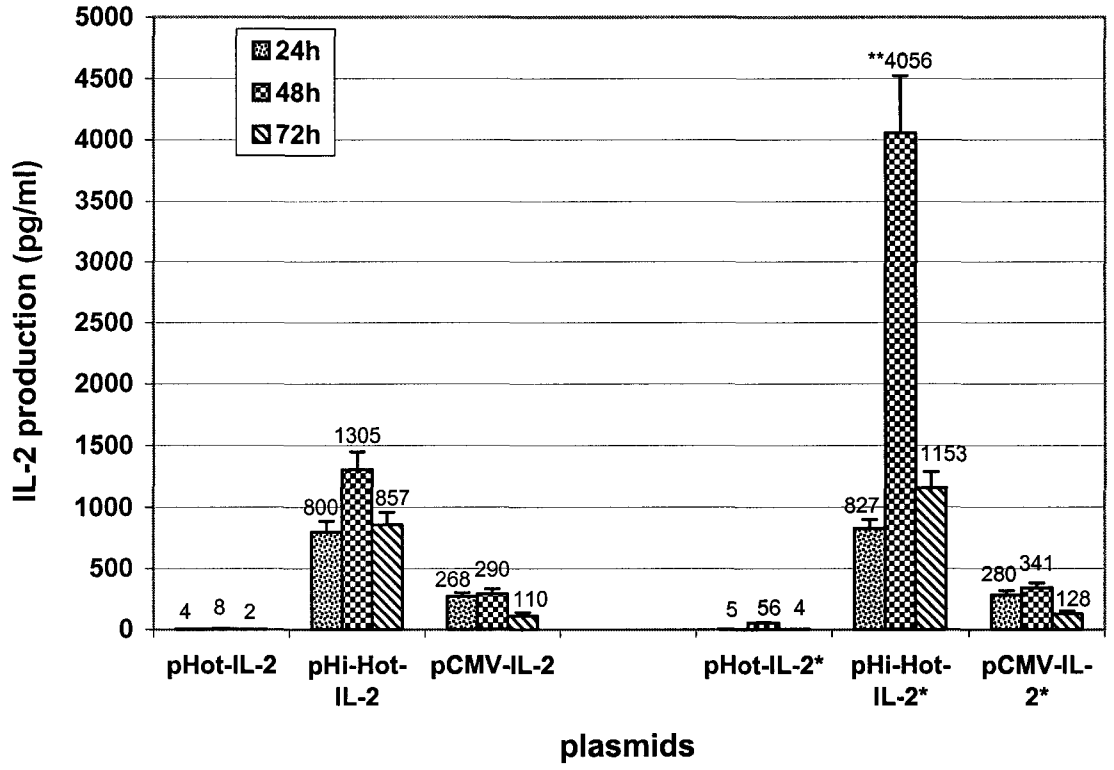


Figure 26. Basal and heat-induced IL-2 production in A549 cells transfected with different plasmids. Cells were heat shocked at 42°C for 30 min 24h post-transfection while controls were kept at 37°C. Supernatants were harvested every 24h for 3 consecutive days. IL-2 production was measured by ELISA. Each bar stands for the IL-2 production in each 24h period after transfection. The values shown are the means of triplicate determinations ± SEM from one of three independent experiments. **p < 0.005 when compared to the IL-2 level of its unheated control (37°C) and to those of the pHot-IL-2 and pCMV-IL-2 24h after heat shock. (*) groups with heat shock treatment

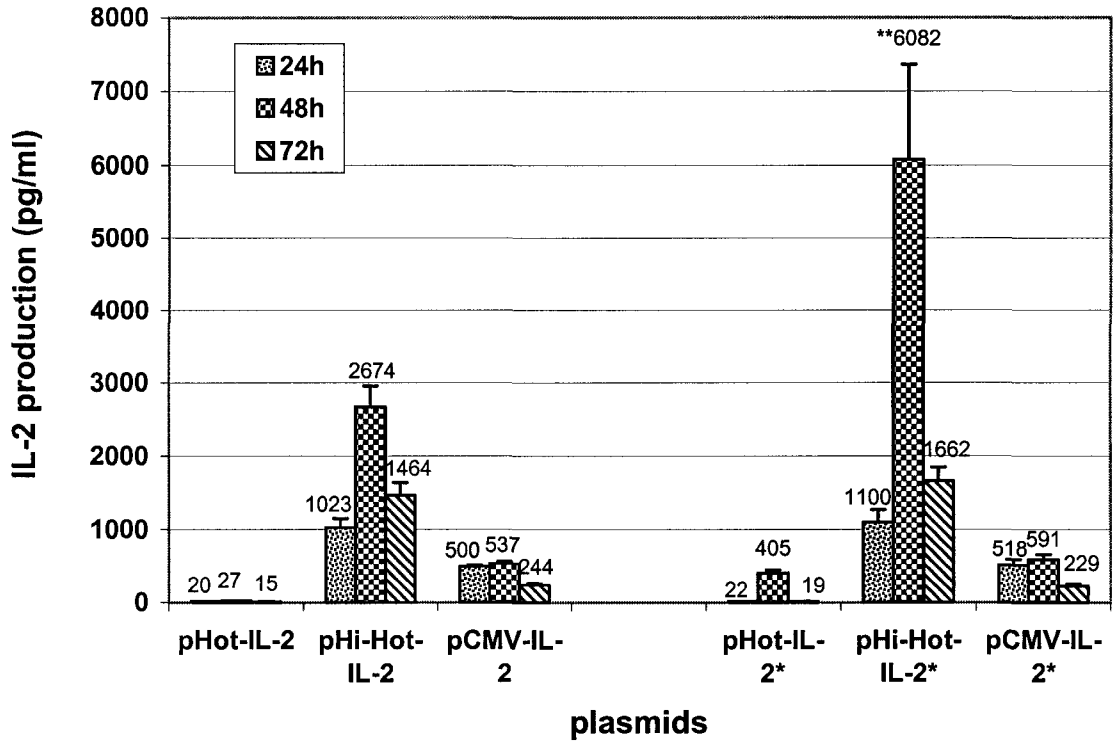


Figure 27. Basal and heat-induced IL-2 production in MCF-7 cells transfected with different plasmids. Cells were heat shocked at 42°C for 30 min 24h post-transfection while controls were kept at 37°C. Supernatants were harvested every 24h for 3 consecutive days. IL-2 production was measured by ELISA. Each bar stands for the IL-2 production in each 24h period after transfection. The values shown are the means of triplicate determinations ± SEM from one of three independent experiments. **p < 0.005 when compared to the IL-2 level of its unheated control (37°C) and to those of the pHot-IL-2 and pCMV-IL-2 24h after heat shock. (*) groups with heat shock treatment

Determination of heat induction of IL-2 secretion from the pHi-Hot-IL-2-nk plasmid in murine B16 and LL/2 tumor cells.

To determine the gene expression profile of the pHi-Hot-IL-2-nk plasmid in murine cell lines, the vector was transfected into mouse B16 and LL/2 cells. The pHot-IL-2-nk and pCMV-IL-2-nk control vectors were also transfected into each cell line. B16 cells transfected with pHi-Hot-IL-2-nk and heated at 42°C for 30 minutes increased transient IL-2 production in the following 24h (48h post-transfection) by 16-fold (24036 ± 3592 pg/ml) as compared to its unheated control (1500 ± 204 pg/ml). The IL-2 levels were 35-times higher than obtained with the pHot-IL-2-nk vector (685 ± 95 pg/ml) and 29-times higher than that from the pCMV-IL-2-nk vector (840 ± 123 pg/ml) (Figure 28). In LL/2 cells, IL-2 production from the pHi-Hot-IL-2-nk vector after heat treatment increased 63-fold (17000 ± 2184 pg/ml) as compared to its unheated controls (270 ± 36 pg/ml). This level of IL-2 production was 36- to 38-times higher than that obtained from using either the hsp promoter (443 ± 61 pg/ml) or the CMV promoter (470 ± 51 pg/ml) directly to drive the IL-2 gene (Figure 29). The CMV promoter responded to heat shock by minimally increasing IL-2 levels 2.4-fold in B16 cells and 6.3-fold in LL/2 cells, as compared to the unheated controls (Figures 28 and 29).

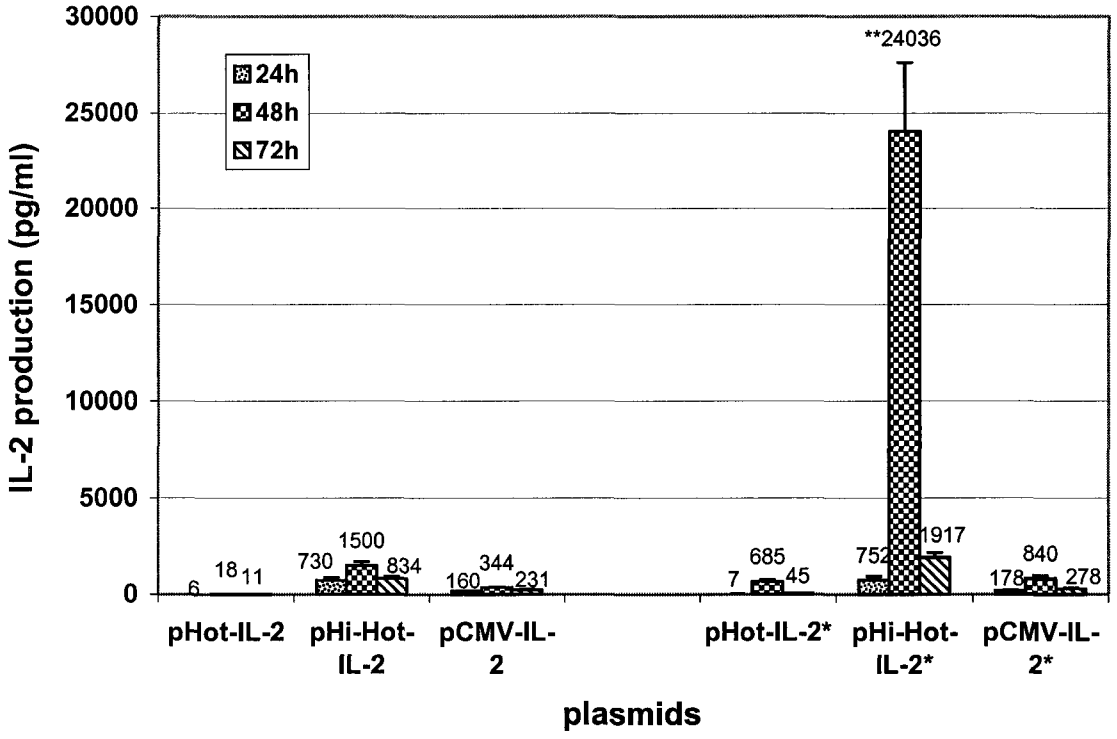


Figure 28. Basal and heat-induced IL-2 production in B16 cells transfected with different plasmids. Cells were heat shocked at 42°C for 30 min 24h post-transfection while controls were kept at 37°C. Supernatants were harvested every 24h for 3 consecutive days. IL-2 production was measured by ELISA. Each bar stands for the IL-2 production in each 24h period after transfection. The values shown are the means of triplicate determinations ± SEM from one of three independent experiments. **p < 0.005 when compared to the IL-2 level of its unheated control (37°C) and to those of the pHot-IL-2 and pCMV-IL-2 24h after heat shock. (*) groups with heat shock treatment

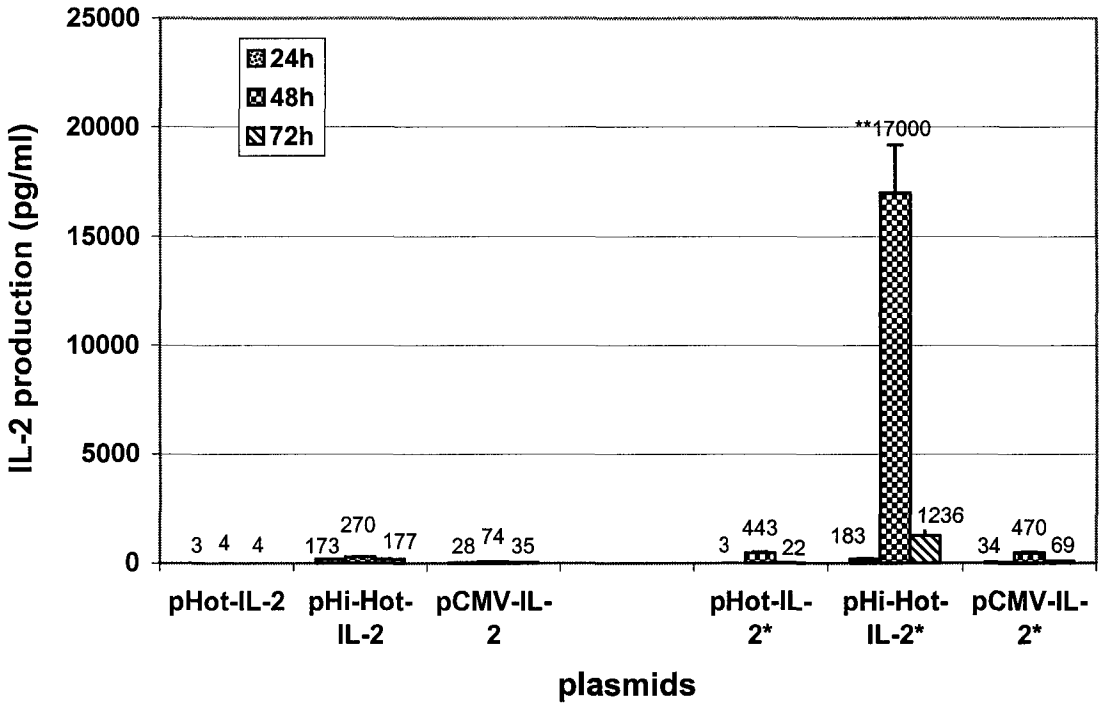


Figure 29. Basal and heat-induced IL-2 production in LL/2 cells transfected with different plasmids. Cells were heat shocked at 42°C for 30 min 24h post-transfection while controls were kept at 37°C. Supernatants were harvested every 24h for 3 consecutive days. IL-2 production was measured by ELISA. Each bar stands for the IL-2 production in each 24h period after transfection. The values shown are the means of triplicate determinations ± SEM from one of three independent experiments. **p < 0.005 when compared to the IL-2 level of its unheated control (37°C) and to those of the pHot-IL-2 and pCMV-IL-2 24h after heat shock. (*) groups with heat shock treatment

Determination of the heat induction of IL-2 secretion from the pHi-Hot-IL-2-nk plasmid in stably transfected LL/2 clones.

To analyze the inducibility of gene expression in stable transfectants, the pHi-Hot-IL-2-nk vector was transfected into LL/2 cells. The pHot-IL-2-nk and pCMV-IL-2-nk vectors were used as controls. Using the previously described protocol, G418-resistant LL/2 clones transfected with each of the three vectors were selected. From each group, one clone with the highest basal IL-2 expression level was chosen for repeated heat shock analysis. To determine the heat induction of IL-2 expression in these stable clones, the cells were heated at 42°C for 30 minutes, at 24h, and again at 72h after seeding into culture wells. Figure 30 shows that each time after heat shock, IL-2 production of the pHi-Hot-IL-2-nk stably transfected LL/2 clones significantly increased over the following 24h (48h: 64543 ± 8030 pg/ml vs 1940 ± 220 pg/ml; 96h: 89504 ± 10738 pg/ml vs 13975 ± 1830 pg/ml) and then dropped back to levels comparable to its unheated control within the next 24h. This finding demonstrated that the pHi-Hot vector retained its heat responsiveness in LL/2 stable clones and that such high gene expression could be multiply induced. Similar to the expression profiles observed with transient transfections, in LL/2 stable clones, heat induced IL-2 production from pHi-Hot-IL-2-nk was 20 to 30-fold higher than that obtained from the pHot-IL-2-nk clones (48h: 2105 ± 175 pg/ml; 96h: 4096 ± 531 pg/ml) (Figure 31) and 75 to 85-fold higher than that from pCMV-IL-2-nk clones (48h: 853 ± 131 pg/ml; 96h: 1017 ± 143 pg/ml) (Figure 32).

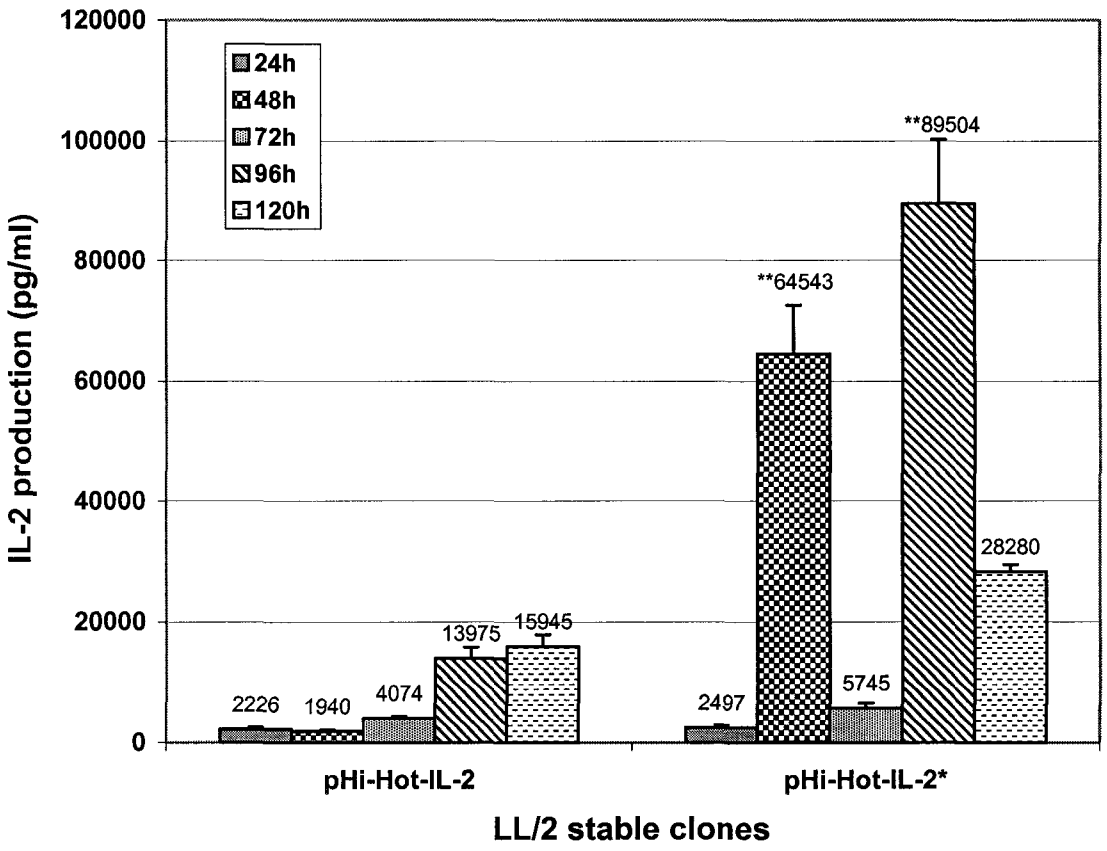


Figure 30. Basal and heat-induced IL-2 production of the pHi-Hot-IL-2-nk plasmid in LL/2 stable clones. LL/2 stably transfected clones were heat shocked at 42°C for 30min at 24h and 72h, respectively after seeding. The unheated controls were kept at 37°C. Supernatants were harvested at each 24h interval. IL-2 production was measured by ELISA. Each bar represents the IL-2 production from the supernatant collected every 24h after plating. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments. **p < 0.005 when compared to its unheated controls (37°C). (*) groups with heat shock treatment

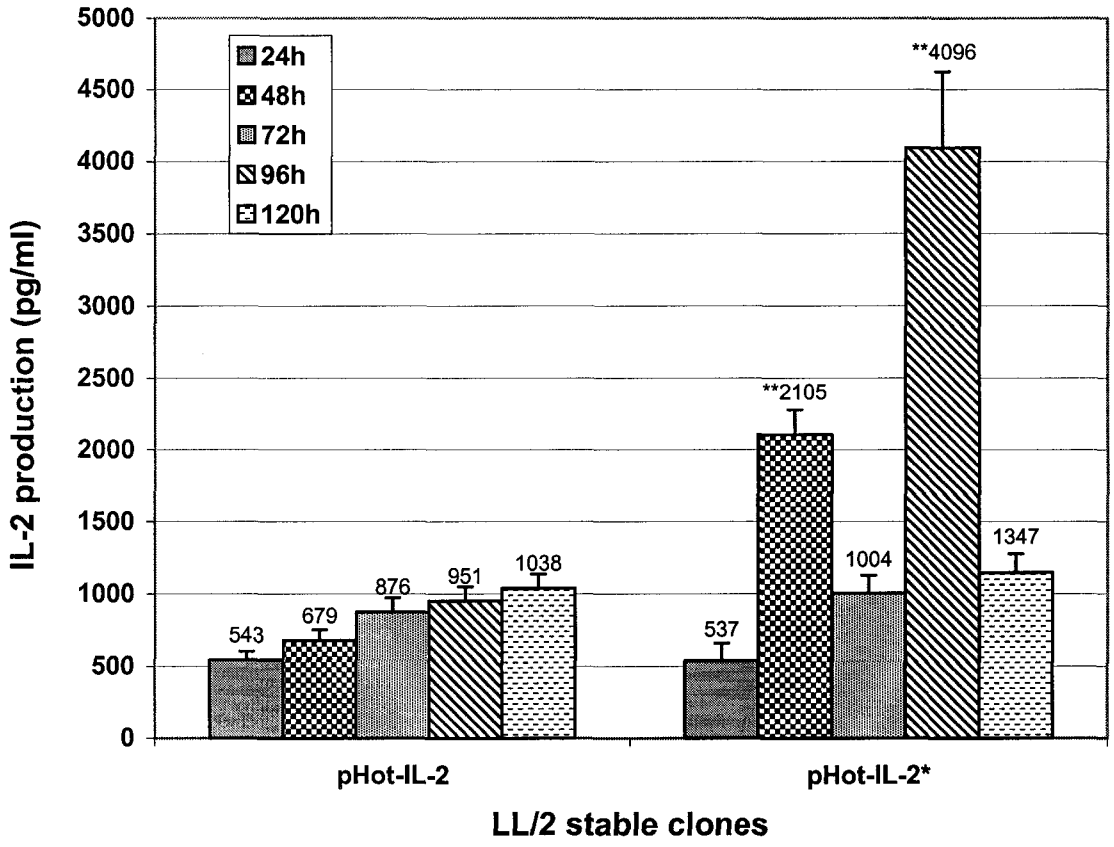


Figure 31. Basal and heat-induced IL-2 production of the pHot-IL-2-nk plasmid in LL/2 stable clones. LL/2 stably transfected clones were heat shocked at 42°C for 30min at 24h and 72h, respectively after seeding. The unheated controls were kept at 37°C. Supernatants were harvested at each 24h interval. IL-2 production was measured by ELISA. Each bar represents the IL-2 production from the supernatant collected every 24h after plating. The values shown are the means of triplicate determinations ± SEM from one of the three independent experiments. **p < 0.005 when compared to its unheated controls (37°C). (*) groups with heat shock treatment

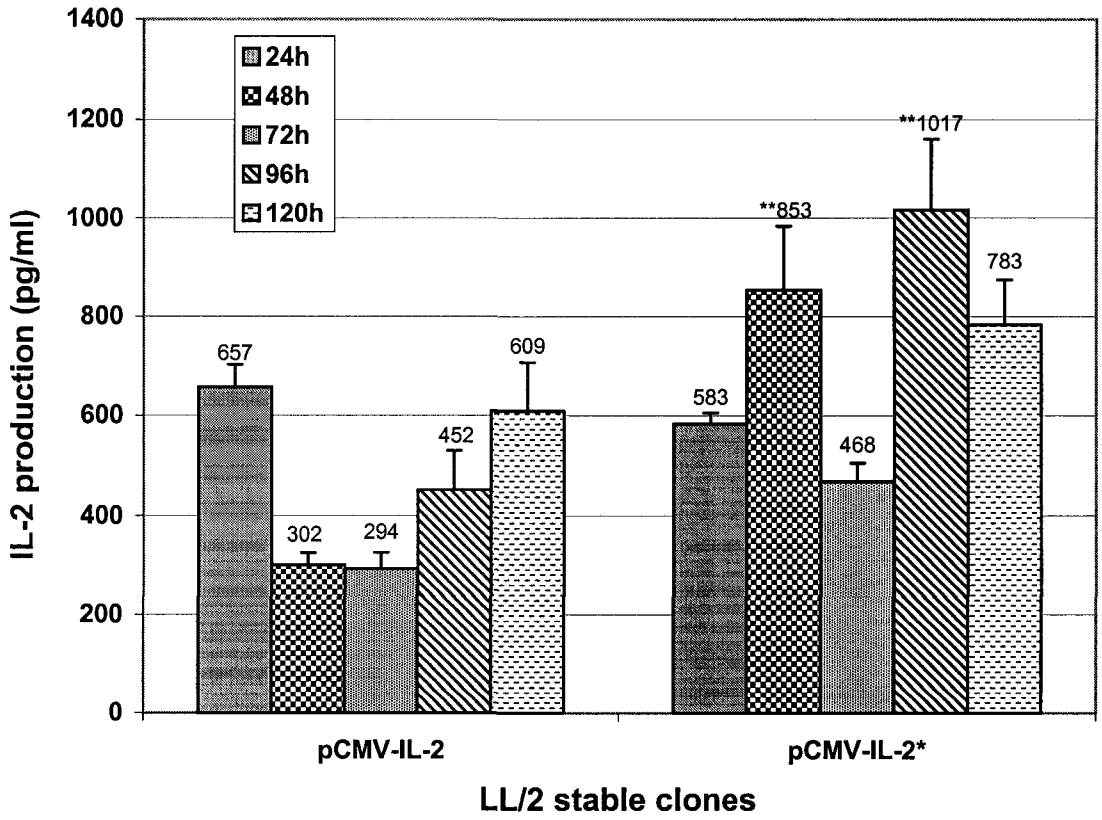


Figure 32. Basal and heat-Induced IL-2 production of the pCMV-IL-2-nk plasmid in LL/2 stable clones. LL/2 stably transfected clones were heat shocked at 42°C for 30min at 24h and 72h, respectively after seeding. The unheated controls were kept at 37°C. Supernatants were harvested at each 24h interval. IL-2 production was measured by ELISA. Each bar represents the IL-2 production from the supernatant collected every 24h after plating. The values shown are the means of triplicate determinations ± SEM from one of the three independnet experiments. **p < 0.005 when compared to its unheated controls (37°C). (*) groups with heat shock treatment

Tumor growth in mice injected with pHi-Hot-IL-2-nk stably transfected LL/2 clones after heat shock treatment.

Having shown that high IL-2 production could be multiply induced in vitro by repeated heat shock in the pHi-Hot-IL-2-nk stably transfected LL/2 clones, we next investigated whether induction of high IL-2 production by repeated heat shock treatment after tumor cell injection could affect tumor growth. Four groups of C57BL/6 mice were used in the preliminary study to investigate the heat tolerance of the mice and any effect of heat shock treatment on tumor growth. Briefly, mice were injected with either parental LL/2 tumor cells or pHi-Hot-IL-2-nk stably transfected LL/2 clones. From each group half of the animals were heated in a waterbath at 42°C for 30 min every other day from day 3 post-injection, whereas the other half was used as a non-heated control. As the heated groups of mice were well tolerant of the 42°C temperature, these mice were then heated at 43°C for 30 min every other day from day 8 post-injection. Tumor development was monitored over an 18-day period (as the heated group of mice injected with parental tumor cells began to die). Figure 33 shows that LL/2 tumors became palpable in all groups of mice 8 days after tumor cell injection. The two groups of mice injected with wild type LL/2 cells had a similar tumor growth rate with or without heat shock treatment. This finding demonstrated that heat shock itself did not affect the tumor growth rate under the experiment conditions. However, tumors started to regress in the heated group of mice injected with the pHi-Hot-IL-2-nk modified clones beginning at day 11, whereas tumors continued to grow

in the non-heated control group. The non-heated group of mice injected with the pHi-Hot-IL-2-nk stable tumor clones showed relatively faster tumor growth as compared to the group of mice injected with the parental tumor cells. This observation could be the result of differential growth rates between individual clones. In this study the growth rates of these clones and the parental cells in vitro was not characterized before in vivo injection. However, multiple heat shock treatment could induce tumor regression in mice injected with the pHi-Hot-IL-2-nk transfected clones. This finding indicated that repeatedly induced high IL-2 expression in these tumor clones led to decreased tumorigenicity in vivo.

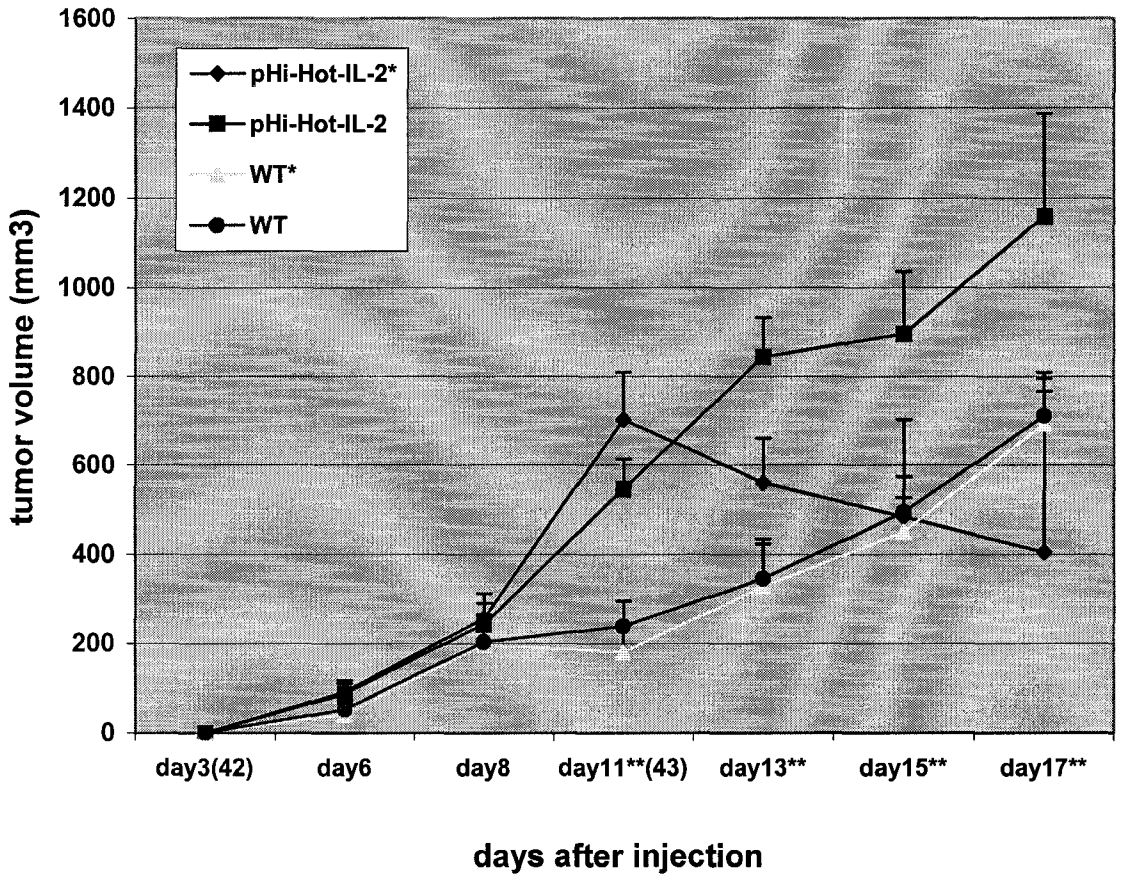


Figure 33. Tumor growth rate in C57BL/6 mice injected with the pHi-Hot-IL-2-nk stably transfected LL/2 clones after heat shock treatment. pHi-Hot-IL-2 genetically modified Lewis Lung (LL/2) clones were injected into C57BL/6 mice. Mice injected with parental LL/2 cells were used as a control. Tumorigenicity of the tumor cells was monitored by measuring the primary tumor growth in mice. Each group contains 4 mice and each mouse was injected with either WT cells or pHi-Hot-IL-2 modified clones subcutaneously at 0.5 x 10^6. Mice from heating groups were put into the waterbath at 42°C for 30min every other day from day 3 after tumor cell injection. Heating temperature was raised up to 43°C for 30min from day 11. Each line represents the average tumor volume ± SEM of 4 mice in each group. (*) group of mice with heat shock treatment. (**) heat shock at 43°C. Data shown are the results from one of two independent experiments.

CHAPTER 5

DISCUSSION

Before gene therapy can deliver on its promise of curing cancer and other diseases, many technical problems need to be solved. As expression vectors are fundamental tools in gene therapy research, one frequently encountered problem during gene therapy vector construction is the lack of compatible restriction sites for DNA insertion. During therapy, efficacy is largely dependent on gene expression levels whereas safety is related to the capability to target and control gene expression. Therefore, the study of gene promoters and the design of gene expression cassettes become more and more important. In this study, a novel user-friendly cloning system, pLinus, was developed to facilitate vector construction. Further, several new strategies were incorporated into vector expression cassettes designed to achieve safe and effective gene therapy.

Features and advantages of the high efficiency pLinus cloning system

The user-friendly pLinus cloning system provides a total of 32 restriction sites for the adaptation of DNA fragments from various sources. DNA fragments with overhangs compatible to only one of the 32 restriction sites in the MCS of the pLinus system can be easily adapted for any of the other remaining 31 sites by first cloning the inserts into one of the pLinus vectors, and then releasing the inserts from any pair of flanking restriction sites.

DNA inserts with two different overhangs compatible to restriction sites in the MCS of the pLinus plasmids can also be adapted to new restriction sites. This task can be accomplished by a complete digestion of the pLinus vector at the inner double-flanking sites followed by a partial digestion of the outer sites. The DNA fragment then ligates into the partially digested pLinus vector. It should be noted that once the DNA fragment is inserted into the pLinus, it has simultaneously been adapted for most of the commonly used restriction sites (such as EcoR I and Hind III), resulting in significant cost-savings. DNA inserts can be further excised and cloned into other expression vectors with any of these restriction sites without needing to repeat the adaptation process, thus saving a considerable amount of time. We have successfully used the Age/Xba I sites of pLinus17-Kpn to adapt the 469-bp SgrA I-Nhe I mouse GM-CSF gene obtained from the pORF-mGM-CSF plasmid (Invivogen, San Diego, CA) for cloning into the Xho I site of an expression vector, pCMV-MCS-neo, constructed by our laboratory (data not shown).

DNA fragments with overhangs incompatible to any of the restriction sites present within the pLinus polylinkers can first be rendered blunt-ended by either exonuclease or DNA polymerase treatment, and then ligated into the blunt-ended site (EcoR V) present in the MCS of each pLinus vector. Once inserted, the DNA fragment can then be excised at the appropriate flanking sites to yield a fragment with cohesive ends of choice. This approach works more efficiently than direct

blunt-end ligation between the DNA inserts and vectors for two reasons: 1) the pLinus plasmids are much smaller than most expression vectors and, 2) once the DNA fragment has been inserted into pLinus, it can be generated with many different cohesive ends for subcloning.

Unmodified PCR products can also be directly adapted for multiple sites using the pLinus cloning systems. In most cases, the naturally occurring compatible restriction sites in PCR fragments are not present. Cloning of the PCR products mainly relies on the TA cloning method (93,94), which exploits the fact that most unmodified PCR products have an A-overhang at the 3'-ends. Such PCR fragments can first be adapted to the commercially available TA cloning vectors before subcloning into the desired vectors or they can be directly ligated to the modified vectors bearing single 3'-T overhangs at both ends (93-97). However, the commercial TA cloning vectors are costly and not all vectors can be modified into T vectors. Each of the four pLinus plasmids can be converted into a T-vector by digestion with the EcoR V enzyme, followed by incubation with Taq DNA polymerase and deoxythymidine triphosphate (dTTP). The advantage of this approach is that the PCR products are simultaneously adapted for all of the most commonly used restriction sites. Such PCR products can then be easily subcloned into other vectors at many different sites.

The potential for adapting DNA fragments of varying size is another significant advantage of the pLinus vectors. One of the problems in introducing restriction sites by PCR is the size limitation for the amplified DNA fragments. Generally, even with high-fidelity polymerases, it is impractical to introduce restriction sites into DNA fragments larger than 20kb by PCR. The size limitation of the pLinus vector for adaptation of large DNA inserts has not been stringently investigated, but the capacity of plasmid vectors to carry DNA fragments as large as 74kb has been reported (98). The pLinus vectors have been used to adapt DNA fragments as small as 100bp (data not shown). Thus, these vectors should be a very useful tool in the field of recombinant DNA technology by making routine manipulation of DNA faster and easier.

High expression vectors using a transcriptional amplifier strategy

In the last few decades, different viral and nonviral-based systems have been developed for gene delivery. Despite many efforts that have been put into improving these systems, one of the major problems facing the gene therapy field continues to be the low expression level of the transgenes (3,8,9). In order to achieve optimal therapeutic efficacy, a high level or at least a threshold level of transgene expression must be reached.

In the case of using nonviral plasmid vectors, one solution has been to increase the delivered dose of DNA using cationic lipids (91,99). However, toxicity from

using high dose of DNA/lipids has limited their practical use in the clinical context (99-104). The development of high expression plasmid vectors would be a possible solution to improve the therapeutic efficacy while reducing the toxicity resulting from using high doses of DNA/lipid.

We therefore devised an "amplifier" strategy to enhance transgene expression by establishing a positive transcriptional feedback loop in a single construct. Two proof of principle vectors, pHi-1 and pHi-2, were therefore constructed. In both the pHi-1/2 vectors, the amplifier expression cassette consisted of two independent transcriptional units in which a transcriptional factor (the tat gene) was driven by a constitutive CMV promoter. The transactivational factor was able to " amplify" the activity of the second promoter (the HIV1/2 LTR) driving the gene of interest. Using IL-2 as a reporter gene, results from transient transfections in two human tumor cell lines showed that both the pHi-1/2 amplifier vectors could achieve significantly higher gene expression levels than that achieved by using the strong CMV promoter alone (Figures 7 and 8). Higher IL-2 production was not caused by a higher transfection efficiency of the amplifier vectors (Figures 12 and 13). Although the transfection efficiency of the two amplifier constructs was much lower than the pCMV control vector in two mouse cell lines tested (Figures 14 and 15), the vectors still produced higher or comparable amounts of IL-2 as compared to the pCMV control vector (Figures 16 and 17). The mechanism for the differential expression and transfection

efficiency profiles noted between the human and mouse tumor cell lines is unknown. However, when tested in vivo, mice injected with pHi-2-IL-2 modified tumor cells had significantly slower tumor growth and longer survival as compared to mice injected with other gene-modified and parental tumor cells. Mice injected with pCMV-pIL-2 modified tumor clones showed only slightly slower tumor growth but not significantly longer survival as compared to control groups (Figures 19 and 20). The difference in tumor growth in the mice was correlated to the serum IL-2 levels obtained after injection with the different vector-modified stable tumor cell clones (Figure 21). It can be assumed that only when IL-2 production from these gene-modified tumor clones reached a certain threshed level could a therapeutic efficacy be reached.

In both human and mouse tumor cell lines tested, the amplifier construct based on the HIV2 LTR produced higher gene expression (IL-2) levels than the HIV 1 LTR. Sequence differences between the HIV1 LTR and the HIV2 LTR have been reported (105). However, the basal promoter activity of the HIV1 LTR and the HIV2 LTR was not compared in the present study. The tat gene encoded in both amplifier vectors was derived from HIVI. However, the exact mechanism by which Tat from HIV I exerted a higher transactivation effect on the HIV2 LTR used in this study is not clear.

High and controlled expression vectors using inducible and amplifier strategies

Control and regulation of gene expression is important for safe gene therapy. Tissue/tumor specific and inducible promoters have been frequently used for such purposes. However, one of the major limitations in using current tissue targeted or inducible expression systems is that the transgene expression level, in most cases, is insufficient for effective therapy (45,47,48). Thus, for safe and effective gene therapy, the development of new expression vectors with high and controlled gene expression properties is desirable.

Combining inducible and amplifier strategies in a single vector, a novel pHi-Hot-MCS-nk plasmid was constructed. Using IL-2 as a reporter gene once again, our data have shown that a moderate heat shock at 42^{o}C for 30 min could drive high gene expression while maintaining the inducible property of the pHi-Hot-IL-2-nk vector. In both human and mouse cell lines, the heat-induced IL-2 expression level of this vector was remarkably higher than that achieved by using either the inducible hsp promoter alone or the strong constitutive CMV promoter directly. By using an inducible hsp promoter to control the expression of the transcriptional activator Tat, the magnitude and duration of the amplified IL-2 gene expression could be regulated by manipulating the activity of the hsp promoter.

In this study, heat shock at 43°C did not result in a significant increase in IL-2 production as compared to heat shock at 42°C in MCF-7 cells (Figure 25). The discrepancy between IL-2 induction and increased temperature/duration probably resulted from the decreased viability of MCF-7 cells after thermal exposure to temperatures higher than 42°C at longer durations. The use of lipid (DMRIE-C)-mediated transfection may decrease the thermal tolerance of these cells, as the nontransfected cells were more tolerant under the same heating conditions. Cell death caused by high levels of IL-2 secretion could be ruled out because much higher IL-2 production obtained in this cell line at these temperatures did not increase cell death when other transfection reagents (i.e., Fugen6) was used (data not shown). In the clinical setting, heating temperatures lower than 43°C should be considered safer and more easily attainable.

Stress-mediated enhancement of transcription has been observed in certain viral promoters, such as the CMV and HTLV promoters (50,106). Thus, it's not surprising that after heat shock, IL-2 production by the pCMV-IL-2 vector increased slightly in the two human cell lines (Figures 26 and 27). Similar results have been reported when using CMV promoter-based vectors in human prostate cancer cells (59). IL-2 production driven by the CMV promoter increased 2.4-fold in mouse B16 cells and 6.3-fold in LL/2 cells after heat treatment (Figures 28 and 29). The reasons for the difference in the magnitude of heat induction of IL-2 by CMV promoter in human versus mouse cells are unknown. It should be noted

that the magnitude of heat-induced IL-2 expression from the hsp promoter was much higher than the CMV promoter in each cell line tested (Figures 26-29).

As the means of controlling the level and duration of gene expression in target tissues have significant implications, promoters with high inducibility and targeted properties are attractive candidates for the development of regulatable gene expression systems. The features of the hsp promoter and the beneficial therapeutic effects of hyperthemia (59,92,107) provide an advantage for using heat inducible systems for such purposes. The hsp promoter responds to many physical and chemical agents (59,92). Previously, we have shown that treatment with heat shock, irradiation or chemotherapeutic drugs could induce gene expression driven by the hsp promoter to levels comparable to that obtained from the CMV promoter (108). This aspect of the hsp promoter provides a flexible choice of modalities to control hsp promoter activity. This fact may be particularly useful for many applications in cancer gene therapy. Hyperthemia has been shown to sensitize tumor cells to radiation, chemotherapy and cytokines in pre-clinical studies (109-112). Radiation and chemotherapeutic drugs can potentially be administered as both inducers and as combined therapeutics in cancer gene therapy regimens. The basal expression level of the hsp promoter is low which can potentially prevent deleterious leakage when vectors carry toxic genes (92). The heat shock response is rapid, but drops quickly after the heat source is removed (59). Such aspects of the hsp promoter in temporal control of gene

expression are advantageous over many other inducible systems, (e.g. the tetracycline system) in which the activating substance cannot be quickly cleared from tissues and blood. As regulation and restriction of gene expression in the target tissue still represents a major challenge in gene therapy (9,54), the development of vectors that can achieve tumor targeting after systemic administration will have a major impact against many common tumor types. The biggest advantage of the hsp promoter is that, even if the vectors are administrated systemically, the expression of the transgene can be targeted within a defined tissue using conformal heating devices. Currently available devices such as microwaves, radiation and ultrasound have made precise, targeted heating achievable in both surface and deep tissues (56,60,113,114). The levels and duration of gene expression can be manipulated by the delivered heat shock (56,59). This fact makes the hsp promoter a good option for targeting purposes, as tissue/tumor specific promoters are not always available (3,46,115). Further, in most cases, there are no means to turn off gene expression from such promoters when necessary (9,11,46). These reasons make the heat inducible gene expression system a promising and feasible approach to targeted and regulatable gene therapy.

The data presented in this study demonstrate that the amplifier strategy is a promising approach to overcoming low levels of gene expression and low efficiency of gene delivery. The advantage of using the amplifier strategy in a

single construct is that the transactivator gene and the gene of interest are always delivered into the same target cells. Given the fact that the inefficiency of DNA nuclear delivery still represents a major barrier in nonviral gene transfer (8,90,116,117), delivering multiple genes simultaneously in a single construct potentially has higher efficiency than delivering each one separately. Such an amplifier strategy could also be very useful in overcoming low gene expression levels when weak cellular promoters need to be used. Combining inducible and amplifier strategies makes it become possible to achieve high and controlled gene expression levels from a single vector.

Future directions

In vivo animal studies using the pHi-Hot-IL-2-nk inducible amplifier vector and a hyperthermia device provided by Thermosurgery Technologies, Inc., Phoenix, AZ will be conducted. Amplifier constructs carrying different therapeutic genes (i.e., GM-CSF) are under construction. Work is also under way to incorporate the amplifier gene expression system into retroviral and adenoviral gene transfer vectors. These derivations should prove to be useful for many laboratories.

Despite the early high expectations for gene therapy, this new therapeutic modality is still in its infancy. Many technical problems in gene therapy need to be resolved before its potential can be fully exploited. Lack of efficient expression and delivery vector systems plus an incomplete knowledge of molecular tumor

pathology represent major hurdles to successful cancer gene therapy. Efforts should be put on developing new strategies to solve these problems. In addition, the existing conventional therapeutic modalities could be combined to further improve therapeutic efficacy.

Use of natural cellular components in vector design.

The current versions of the pHi-1/2 amplifier vector and the pHi-Hot inducible amplifier vector use a Tat transcriptional factor and the HIV LTR promoter to demonstrate the principle that increased transgene expression levels can be achieved by incorporating the amplifier strategy into a single construct. As many transcriptional factors and promoters have been identified and characterized, different combinations of transcriptional factors and augmentable promoters can now be incorporated into vector constructions for different purposes. Substitution of the viral transactivator in the vector with natural cellular components could be advantageous. The use of natural cellular components as regulators may circumvent problems for in vivo gene therapy due to immunogenic responses or toxic side effects (118-121). New version amplifier vector using heat shock factor (HSF) and hsp promoter is under construction.

Development of polycistronic vectors for synergistic and multi-purpose uses.

Many studies have shown that improved therapeutic efficacy can be achieved by

using multiple genes that synergize (122,123). Development of gene therapy vehicles capable of delivering multiple genes is desired. This goal can be accomplished by designing multi-gene expression cassettes that use independent transcriptional units in a single construct. Previously, we have constructed several plasmid vectors that use multiple independent transcriptional units to co-express two reporter genes. We have shown that two genes could be efficiently co-expressed in these vectors without compromising transfection efficiency as compared to using the same plasmid carrying a single gene (data not shown). One of the biggest advantages of using a plasmid vector is its capacity to introduce large pieces of DNA. Development of plasmid-based polycistronic vectors may be very useful either to co-express several proteins for a combined/synergistic effect or form a heteromultimeric protein, and to reconstruct a metabolic pathway. Very complex and time-consuming co-introductions of several genes can be shortened using polycistronic vectors (124).

The use of multiple independent transcriptional units in an expression cassette is advantageous over the use of a viral IRES sequence in that, when the expression levels of the genes upstream and downstream of the IRES are compared, the levels of expression of the gene downstream of the IRES is typically 10-50% of that of the upstream gene (87,88). These values can vary depending on cell types and reporter genes used (125). These observations

mean that protein expression levels directed by an IRES cannot be reliably predicted. Another disadvantage of the IRES is the relatively large size (about 0.5 kb), similar or longer than commonly used promoters such as CMV or SV40. In addition, in comparison to promoters, there is no known type of regulation or specificity of IRES elements (125). These facts limit its use in developing regulatable gene expression systems.

Vector optimization to achieve high and longer transgene expression levels in vivo.

Transient or gradual loss of transgene expression still remains a problem in gene therapy regardless of the gene delivery system (8,9,119,120). Sustained gene expression is also an important goal for nonviral gene therapy. Controllable integration of plasmid DNA into the genome may possibly provide long-term gene expression. Incorporation of a transposon, and several phage integrases (and their corresponding recognition elements) has been reported to increase intergration (126,127). Although the efficiency is still low, these technologies provide new directions and possible solutions and need to be further improved. On the other hand, host immune response elicited by delivered vector components can play an important role in determining the duration of gene expression (14,20,21,121,128). To achieve longer transgene expression, particular care must be taken to avoid immunogenic vector components when designing an expression system to be used for gene therapy protocols. Plasmid

DNA vectors capable of persistent transgene expression without the need for any viral proteins would also be desirable. This goal may be realized by using cellular transcriptional factors that prevent promoter inactivation, or by identifying constitutively active cellular promoters.

Combination of genes with synergy and/or use with conventional therapy.

Tumor heterogeneity makes it unlikely that a single therapeutic approach will prove effective for all tumors. Combined treatment with synergistic cytokine genes has augmented the antitumor effect in different murine tumor models (129,130). Potentiation of therapeutic efficacy can be achieved by co-treatment of animals with cytokines such as GM-CSF, IL-2 and IL-12. Future therapeutic approaches will probably rely on combinations of immune adoptive therapy, molecular chemotherapy and/or prodrug conversion that also protect normal cells during treatments.

Increasing the efficiency of DNA delivery.

Inefficient entry of DNA into the nucleus of target cells is a major limiting step in the development of nonviral gene delivery systems. In vivo, DNA delivery by electrophoration is a gene delivery technique that has been used successfully for efficient delivery of plasmid DNA to many different tissues (131). Delivery of plasmids into the tumor by electrophoration may enhance plasmid DNA uptake in tumor tissue, resulting in increased overall gene expression levels within the

tumor (131-133). Other strategies such as ultrasound and nuclear localization signal peptides have been used to increase active DNA transport into the nucleus (134,135).

We have a long way to go in terms of understanding the barriers between vector delivery and cell biology. Although the theoretical advantages of gene therapy are indisputable, convincing clinical efficacy has not yet been demonstrated in most of the trials conducted to date. However, in recent years, the results of some published phase I and II cancer gene therapy studies are encouraging (63,64,136-138). Important progress in vector technology is expected in the near future. A combination of vector biology, immunology and cell biology will be needed to develop better vector systems for successful gene therapy.

CHAPTER 6

SUMMARY AND CONCLUSION

In this study, using classical *in vitro* ligation/restriction techniques, two sets of cloning plasmids (pLinus16/17) were constructed. With its flexibility in the choice of 32 unique sites for the most commonly used restriction enzymes, the pLinus cloning system can provide highly efficient and versatile adaptation for routine cloning and manipulation of DNA fragments from various sources. Saving both time and resources, it can greatly facilitate vector construction. This system should be particularly useful for those individuals who do not have easy access to all of the sophisticated modern cloning technologies.

Effective gene therapy often depends on sufficient expression levels of the transgene. One of the major obstacles in gene therapy currently is the low transgene expression from the available vector systems. To address this issue, a transcriptional amplifier strategy was incorporated into a single construct. Two proof of principle vectors (pHi-1-MCS and pHi-2-MCS) were constructed. In these amplifier vectors, a constitutive CMV promoter was used to drive the expression of a transcriptional factor, the tat gene, which could transactivate a second promoter (the HIV1/2 LTR) located in the same construct. The second promoter then controlled the gene of interest. Using the human IL-2 cytokine gene as a reporter gene, our data demonstrated that the pHi-1/2 amplifier vectors could

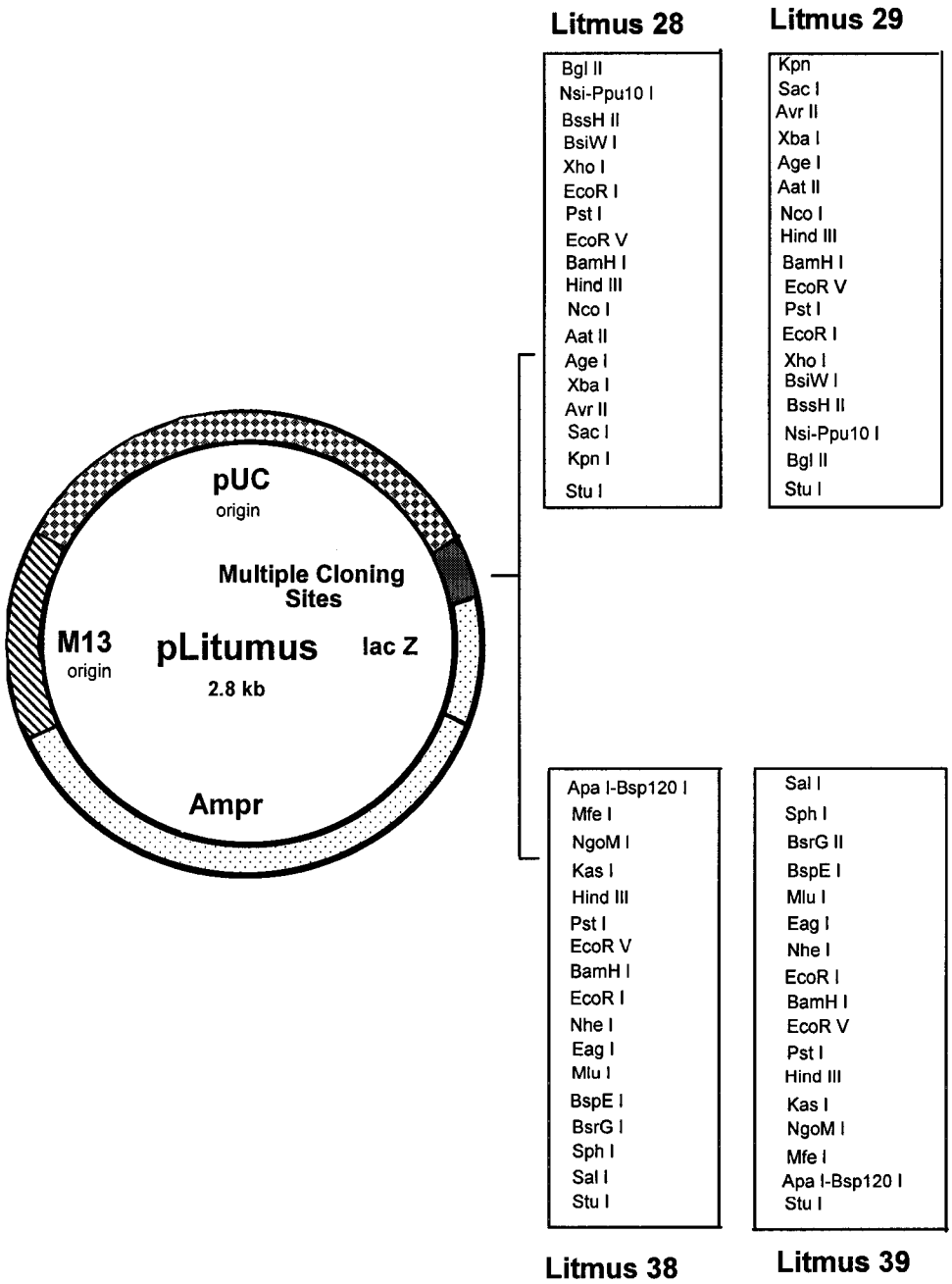
achieve significantly higher IL-2 levels in human tumor cell lines than that obtained from using the CMV promoter alone. When tested in vivo using a murine LL/2 tumor model, injection of pHi-2-IL-2 modified tumor clones led to slower tumor growth and longer survival as compared to using clones stably transfected with pCMV-IL-2, vector backbone transfected clones and parental LL/2 tumor cells. Thus, the amplifier strategy when incorporated into gene therapy vectors may greatly improve their efficacy by increasing transgene expression levels.

As safety is equally important in evaluating any practical gene therapy protocol, development of high but controlled transgene expression is often required for safe and effective gene therapy. Based on the pHi-2-MCS amplifier vector, a novel inducible and amplifier vector (pHi-Hot-MCS-nk) was therefore constructed in which the constitutive CMV promoter was replaced by an inducible hsp promoter. Using human IL-2 cytokine gene again, in both human and mouse tumor cell lines, the pHi-Hot-IL-2-nk vector demonstrated high IL-2 expression levels while maintaining its inducibility after moderate heat shock at 42°C. High and inducible expression was repeatable in LL/2 stably transfected clones after multiple heat shock treatments. Our preliminary data showed that multiple heat shock treatments at 42°C led to slower tumor growth in mice after injection of pHi-Hot-IL-2-nk modified tumor clones as compared to a control group without heat shock treatment and a control group injected with parental LL/2 tumor cells.

Heat shock treatment did not affect primary tumor growth in mice injected with parental LL/2 tumor cells.

These experiments showed that transgene expression levels driven by promoters with moderate or strong activity could be further enhanced by incorporation of an amplifier strategy. Further, high and controllable transgene expression could also be achieved by incorporation of both inducible and amplifier strategies into a single vector. In our experience, no other promoter to date could achieve comparable transgene expression levels in these cell lines. Especially for nonviral-based expression systems, this approach provides a possible and practical method to overcome low gene delivery efficiency. Taken together, these results indicated that improved expression levels and therapeutic efficacy could be achieved by incorporating novel strategies into expression cassette design. These strategies provide important directions for the development of new expression vectors for safe and effective gene therapy.

APPENDIX A


Litmus 28
Litmus 29
Bgl II
Nsi-Ppu10 I
BssH II
BsiW I
Xho I
EcoR I
Pst I
EcoR V
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Hind III
Nco I
Aat II
Age I
Xba I
Avr II
Sac I
Kpn I
Stu I
Kpn
Sac I
Avr II
Xba I
Age I
Aat II
Nco I
Hind III
BamH I
EcoR V
Pst I
EcoR I
Xho I
BsiW I
BssH II
Nsi-Ppu10 I
Bgl II
Stu I
pUC
origin
Multiple Cloning
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pLitumus
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lac Z
Ampr
Apa I-Bsp120 I
Mfe I
NgoM I
Kas I
Hind III
Pst I
EcoR V
BamH I
EcoR I
Nhe I
Eag I
Mlu I
BspE I
BsrG I
Sph I
Sal I
Stu I
Sal I
Sph I
BsrG II
BspE I
Mlu I
Eag I
Nhe I
EcoR I
BamH I
EcoR V
Pst I
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Mfe I
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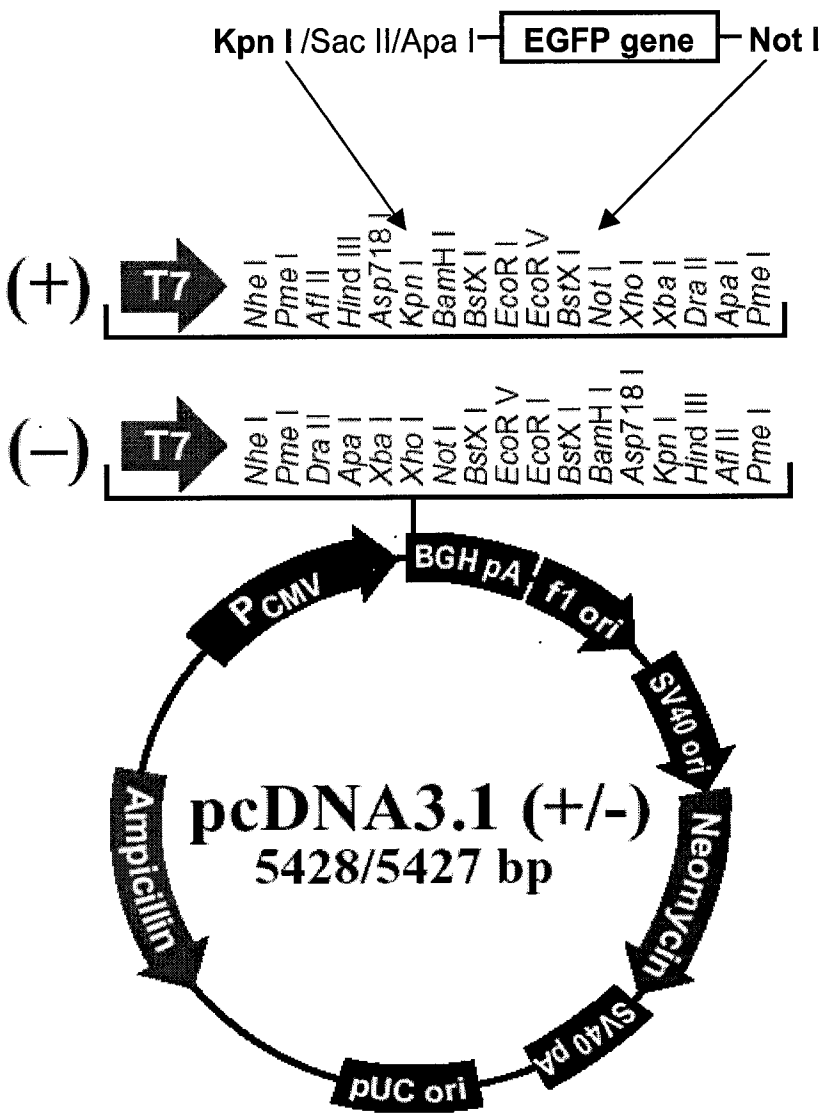
Stu I
Litmus 38
Litmus 39

APPENDIX B

pcDNA3.1-EGFP



APPENDIX C

Buffers and Media

Complete RPMI

To 500ml of RPMI (Scientific Irvine, Santa Ana, CA) add:

50ml of Heat Inactivated Fetal Bovine Serum (Gibco-BRL, Grand Island, NY)

5ml of 200mM L-glutamine (Gibco-BRL, Grand Island, NY)

5ml of 1000u/ml Penicillin + 1000ug/ml Streptomycin (Gibco-BRL, Grand Island, NY)

5ml of 100mM sodium pyruvate (Gibco-BRL, Grand Island, NY)

5ml of 100mM Non-essential amino acids (Gibco-BRL, Grand Island, NY)

250ul of 5mg/ml of Getamycin (Sigma, St. Louis, MO)

5ul of B-mercaptoethanol (Sigma, St. Louis, MO)

Freeze Medium

40% Fetal bovine serum (FBS) (JRH Biosciences)

50% α-IMDM

10% Dimethy Sulfoxide (DMSO) (Fisher Scientific, Pittsburgh, PA)

1% Paraformaldehyde FACS Fixative

500ml of 1X PBS w/o Ca, Mg

5g Paraformaldehyde powder

pH with:

-NaOH pellets

-HCl. 1N solution

50X TAE

242.0g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA, pH 8.0

Luria Broth (LB) (pH 7.0)

1L volume added with:

10.0g NaCL

10.0g Tryptone

5.0g yeast extract

SOC Medium

5.0g Tryptone

1.25g yeast extract

0.15g NaCl

0.05g KCl

245 ml H2O, pH to 7.0

Autoclave and add the following:

2.5 ml 2 M Mg+ solution (1M MgSO4, 1M MgCl2)

2.5 ml 2M glucose

APPENDIX D

Viability Staining Protocol

Determination of cell viability was performed by staining cells with Trypan Blue Dye (Sigma immunochemicals, St. Louis, MO) according to the following protocol:

450 ul of Trypan Blue Dye was mixed with 50ul of the cell suspension. A 10ul aliquot of the cell-dye mixture was loaded onto a hemocytometer (American Optical Scientific Instruments, Buffalo, NY) for counting under a light microscope (Cal Zeiss Inc, San Leandro, CA).

Cell concentration was calculated according to the formula:

$$\text{Cells /ml} = \frac{\text{number of cell counted} \times 10^4 \times \text{dilution factor}}{\text{number of squares counted}}$$

Percentage cell viability was calculated according to the formula

$$\%\ \text{cell viability} = \frac{\#\ \text{of total viable cells} \times 100}{\text{total \# of cells}}$$

APPENDIX E

Animal approval form

Institutional Animal Care and Use Committee

THE UNIVERSITY OF ARIZONA
TUCSON ARIZONA

P.O. Box 210101
Tucson, Arizona 85721-0101

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted *Final Approval* according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:

~~#00-105 - "Gene Therapy of Cancer"~~

PRINCIPAL INVESTIGATOR/DEPARTMENT:

David T. Harris, PhD - Microbiology & Immunology

GRANTING AGENCY:

Cord Blood Bank Discretionary Funds

SUBMISSION DATE: June 6, 2000

APPROVAL DATE: November 8, 2000 APPROVAL VALID THROUGH*: November 7, 2003

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: November 9, 2000

REVISIONS (if any):

MINORITY OPINIONS (if any):

Richard C. Powell

Richard C. Powell, PhD, MS
Vice President for Research

DATE: November 9, 2000

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.

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